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<b>14. ABSTRACT</b> The purpose of the Breast Cancer Research Training Program is to recruit and train potential future researchers in breast cancer research. During the 3-year grant, we recruited 75 applicants to the program, interviewed 22 candidates, and hired 15 students. Each year, students attended up to 7 research lectures, participated in weekly meetings, developed and carried out research projects under the supervision of their mentors, and presented their projects to a research audience. Projects conducted over the course of the grant built upon previous years' research. Ongoing research was conducted on the anticancer properties of an Asian mushroom, Ganoderma lucidum, upon highly invasive breast cancer cells, on the role of omega-3 fatty acids in preventing and treating breast cancer, and in the mechanisms by which C-reactive protein exerts its anticancer properties in breast cancer. Other projects addressed the effect of kinase inhibition on ERK activity in breast cancer cells, the role of the calpain proteolytic pathway in breast cancer-induced cachexia, and the inhibitory effects of a Japanese mushroom, Phellinus linteus, in breast cancer cells. These results have added significant new information to our understanding of dietary modulation of breast cancer and have provided 15 students with experience in breast cancer research.					
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## **Introduction**

The Breast Cancer Research Training Program at the Methodist Research Institute at Clarian Health was funded by the US Army Medical Research and Materiel Command and for the period 4/15/2003 to 4/14/2006. The purpose of the Breast Cancer Research Training Program was to recruit, train, and provide the opportunity to well-qualified college science students to work on a biomedical research project in breast cancer with a medical researcher in the area of breast cancer. Our goal was to instill in the students a passion for and commitment to breast cancer research so that they might take up breast cancer research as a career. This training was accomplished during the months of May through August each year.

This final report presents the accomplishments of each program by year. The body of the reports for each year lists the students and preceptors, and describes the training accomplishments for each year based on the statement of work. The next section lists research accomplishments, describing each of the five breast cancer research projects undertaken by the students. The final section defines the reportable outcomes of the program, especially published manuscripts and presentations. The appendix includes reprints of the reportable outcomes material for each year.

### **2003 Breast Cancer Research Training Program**

#### **Body**

Planning for the 2003 Breast Cancer Research Training (BCRT) program began in November 2002 with development of application materials and the start of recruitment procedures. The application due date was in mid-February 2003 and students were offered positions in late March to early April. Students began the program the 3<sup>rd</sup> week of May and the program ended the 2<sup>nd</sup> week of August with Presentation Day.

Fifteen students were recruited to the 2003 BCRT program. Four of the applicants were from underrepresented minorities. We interviewed eight candidates for five positions in the program.

The following list presents the students who were ultimately chosen, the college or university attended and class status at the time of application, and their BCRT program preceptor:

<b>Student</b>	<b>Class and Institution</b>	<b>Preceptor</b>
Justin Bell	Junior, Purdue University	Daniel Sliva
Phillip Boykin (minority)	Junior, Rochester Inst of Tech	Frank P. Lloyd, Jr.
Melissa Cain	First Year, Indiana Univ Schl of Med	Carlos A. Labarrere
Laura Sech	Junior, University of Notre Dame	Rafat A. Siddiqui/Gary P. Zaloga
Heidi Yount	Junior, Ohio Northern University	Rafat A. Siddiqui

## 2003 Training Accomplishments According to the Statement of Work

### Task 1: Recruitment of undergraduate students interested in breast cancer research training

- November 18, 2002: Program application updated and information materials prepared for website.
- November 18 to November 30, 2002: Information packages with application materials to students, colleges/universities, and community leaders mailed.

*Deviation:* We had proposed to make follow-up phone calls to college/university biomedical departments and career centers, and to community leaders emphasizing the breast cancer research program; however, at the time this grant was awarded, we had already recruited the candidates for all available positions. Therefore, follow-up calls were not made for the 2003 program. This task received greater priority with subsequent programs.

### Task 2. Selection of students to participate in the Breast Cancer Research Training Program

- February 14, 2003: All applications received by February 14, 2003 or with a postmark of February 14, 2003 were considered for the program.
- The Selection and Interviewing Committee chose prospective candidates and interviewed them.

*Deviation:* We originally proposed to have preceptors interview students. Since that time, the Program Director and the Program Coordinator decided that rather than the preceptors doing the interviewing, a Selection and Interviewing committee would interview the students. In the past, preceptors had been given three students to interview and would interview the first without interviewing the other two. To create a fairer system in which all students selected for interviewing would have a chance to demonstrate their knowledge and enthusiasm, a committee was convened. For the 2003 program, three of the committee members were faculty on the BCRT project. Interviews were conducted during the 11 days from March 11, 2003 through March 22, 2003.

- March 22, 2003: Interviews with students were completed and committee members chose five students to participate in Breast Cancer Research Training Program and matched them with BCRT preceptors.
- March 24 – March 31, 2003: Selected students were notified of their acceptance to the program.

*Deviation:* Because we had already recruited the candidates for the positions before the grant was awarded in April 2003, we had a medical student participate in the program the first year of the grant.

Task 3:        Breast Cancer Research Training Program

- May 19, 2003: Students began the Methodist Research Institute Breast Cancer Research Training Program.
- Students attended seven lectures between May 30, 2003 and July 18, 2003.
- From May 21, 2003 to August 6, 2003, students conducted breast cancer research under the guidance of their preceptors. This training included weekly meetings of all student and preceptor participants in the program.
- Students attended a lecture on writing research papers; preceptors guided their students in writing their research papers.
- Preceptors guided students in their preparation of oral presentations; students presented their projects informally to other students at weekly meetings.
- August 7, 2003: All students participated in rehearsal for Presentation Day.
- August 8, 2003: All students delivered oral presentations of their projects to an audience of researchers at Methodist Hospital.
- August 8, 2003: All students turned in their research papers.

Task 4.        Evaluation of the Program

- August 12, 2003: Students turned in evaluation forms for the program.
- August 20, 2003: Program Coordinator wrote a final evaluation of the Breast Cancer Research Training Program.
- November 2003: Program Director and Program Coordinator met to review the evaluation.
- Program Director and Program Coordinator developed plans for improvement of the program based on the evaluation.

*Issues Raised.* Two students reported that they needed more attention from their preceptors.

*Recommendations.*

- Continue to stress in the organizational meeting for preceptors the importance of availability and attentiveness of preceptor.
  - Schedule vacations at non-critical times. Avoid start of program and Presentation Day.
- Invite a successful preceptor to the orientation meeting to present some insight on what it takes to be a good preceptor.

*Implementation.* A preceptor who is considered particularly able and successful presents at the annual organizational meeting. One of the preceptors has since left the program and been replaced with another preceptor.

## Key Research Accomplishments for the 2003 Program

### Research Accomplishments

The following paragraphs describe the projects undertaken in the 2003 Breast Cancer Research Training Program and the results of those projects.

#### Project 1. *Ganoderma lucidum* displays antiestrogenic activity in both MDA-MB-231 and MCF-7 breast cancer cells.

Justin Bell and Dr. Daniel Sliva, Cancer Research Laboratory at the Methodist Research Institute, investigated the anticancer properties of an ancient Asian medicinal mushroom, *Ganoderma lucidum*. The purpose of this project was to determine whether *Ganoderma* displays estrogenic or antiestrogenic activity in highly invasive breast cancer cells (MDA-MB-231) and in less invasive MCF-7 breast cancer cells. The study found that *Ganoderma lucidum* inhibits estrogen receptor signaling in both MCF-7 and MDA-MB-231 breast cancer cells.

#### Project 2. Breast cancer cells treated with *Ganoderma lucidum* show changes in gene expression.

Phillip Boykin and Dr. Frank P. Lloyd, Jr., Surgical Oncology, Methodist Hospital, also investigated *Ganoderma lucidum*. The objective of their project was to use microarray analysis to determine whether breast cancer cells treated with *Ganoderma* show differences in gene expression. Results were inconclusive because of RNA degradation after isolation; however, future plans intend to focus more on RNA isolation in preparation for microarray analysis.

#### Project 3. The effects of C-reactive protein treatment on uPA and uPAR production by highly invasive breast cancer cells.

Melissa Cain and Dr. Carlos A. Labarrere, Experimental Pathology Laboratory at Methodist Research Institute, examined whether C-reactive protein (CRP), an acute-phase protein with immunological functions, promotes cell growth in highly invasive MDA-MB-231 breast cancer cells through upregulation of urokinase plasminogen activator (uPA) and its receptor (uPAR). Their study found that CRP promotes increased secretion of uPA by highly invasive breast cancer cells and promotes upregulation of uPAR on the cell membranes of highly invasive breast cancer cells.

#### Project 4. Analysis of commercial fish oil supplements and their efficiency in inhibiting metastasis and promoting apoptosis of breast cancer cells.

Laura Sech and Drs. Rafat A. Siddiqui and Gary P. Zaloga, Cellular Biochemistry Laboratory at Methodist Research Institute, studied the anticancer effects of omega-3 fatty acids in fish oil. Ms. Sech's project examined seven commercial preparations of omega-3 lipids for differences in chemical composition, quantity of omega-3 fatty acids, and their anticancer activity in MDA-MB-231 breast cancer cells. Her analysis found that significant compositional differences exist between the different preparations and that the different preparations exhibited widely variable anticancer effects. This study also found that fatty acid methyl esters had the most potent anticancer effects.

**Project 5. Role of omega-3 fatty acids in the prevention of cancer-induced muscle proteolysis.**

Heidi Yount and Dr. Rafat A. Siddiqui also studied omega-3 fatty acids (O3FAs). The hypothesis of the study was that O3FAs, primarily docosahexaenoic acid, provide protection against tumor-induced muscle wasting by inhibiting activation of calpains. The results of the study suggest that MDA-MB-231 breast cancer cells release soluble proteolysis-inducing factors that induce proteolysis by activating caplain enzymes. Omega-3 fatty acids inhibit the calpain-associated proteolysis pathway. Thus, omega-3 lipids may help prevent body wasting associated with cancer.

**Reportable Outcomes for the 2003 Program** (Students' names are in bold.)

**Manuscripts:**

Preceptors: Rafat Siddiqui and Gary Zaloga

Siddiqui R, Shaikh S, **Sech L, Yount H**, Stillwell W, Zaloga G. Omega-3 fatty acids: health benefits and cellular mechanisms of action. *Minirev Med Chem.* 2004;4:859-871.

**Presentations:**

Preceptor: Rafat Siddiqui

**Yount H**, Siddiqui R. Role of omega-3 fatty acids in the prevention of cancer-induced muscle proteolysis. 227th ACS National Meeting, Anaheim, CA. March 27 - April 1, 2004, Abstract #CHD242.

**Conclusions for 2003 Breast Cancer Research Training Program**

We successfully recruited 15 applicants, interviewed eight candidates, and selected five students to participate in the 2003 Breast Cancer Research Training Program at the Methodist Research Institute. Trainees attended a series of lectures dealing with research design, statistics, ethics, and research reporting. Trainees developed and worked on individual research projects under direct supervision of a mentoring researcher. Each trainee significantly contributed to larger research projects dealing with breast cancer. Four of the five projects dealt with dietary modulation of breast cancer proliferation and invasion, and involved evaluation of the effects of Ganoderma lucidum (a mushroom) and omega-3 long-chain fatty acids. Both of these compounds have been found to have anticancer activities in previous studies at the institute. The fifth project evaluated the effects of C-reactive protein, a proinflammatory compound, upon breast cancer cells. The knowledge obtained in these studies contributes to our knowledge of dietary modulation of breast cancer cell growth and invasion. These results helped determine future areas of research.

**2004 Breast Cancer Research Training Program**

**Body**

Planning for the 2004 Breast Cancer Research Training Program began in November 2003 with development of application materials and student recruitment. The application due date was February 17, 2004, and students were offered positions in late March to early April. Students began the program on May 24<sup>th</sup> and the program ended August 13<sup>th</sup>. Presentation Day was moved up to August 6 because students entering medical

school needed to leave the following week for orientation to their medical school programs.

The paragraph below is modified from the 2004 annual summary after discrepancies were pointed out in a review by the Army MRMC.

Twenty-four students applied to the 2004 Breast Cancer Research Training Program. Three of the applicants to the program were from underrepresented minorities. We interviewed seven candidates for two positions in the program (two students from the 2003 BCRT program returned for a second year in the 2004 program and one student from the general Summer Student Research Program in 2003 was accepted into the 2004 Breast Cancer Research Training Program).

We invited students to return to the program for a second year based on our conviction that students who are interested enough to repeat the program should be encouraged in their interest. Furthermore, other agencies that fund undergraduate and medical student research training program encourage hiring students for at least two consecutive years to enhance the research training experience. A repeat year provides the opportunity to build upon students' knowledge and continue their formation in breast cancer research.

The following list presents the students who were ultimately chosen, the college or university attended and class status at the time of application, and their BCRT program preceptor(s):

<b>Student</b>	<b>Class and Institution</b>	<b>Preceptor</b>
Brian Bock	Junior, Valparaiso University	Daniel Sliva
Jennifer Griffith (returning from SSRP)	Senior, Indiana University	Thomas Kovala
Laura Sech (returning)	Senior, University of Notre Dame	Rafat A. Siddiqui/Gary P. Zaloga
Chelimo Yego (minority)	Senior, Eastern University	Carlos A. Labarrere
Heidi Yount (returning)	Senior, Ohio Northern University	Rafat A. Siddiqui/Gary P. Zaloga

Three of the students were accepted into medical school during the course of the summer.

#### 2004 Training Accomplishments According to the Statement of Work

##### Task 1: Recruitment of undergraduate students interested in breast cancer research training

- October 2003: Program application updated and information materials prepared and uploaded onto the website
- October through November 2003: Information packages with application materials to students, colleges/universities, and community leaders mailed. Application packages and brochures were mailed out in response to requests for applications.

- December 2003: Follow-up phone calls were made and brochures were sent out to promote the Breast Cancer Research Training Program at Methodist Research Institute.

Task 2.        Selection of students to participate in the Breast Cancer Research Training Program

- February 17, 2004: All applications received by February 17, 2004 or with a postmark of February 17, 2004 were considered for the program.
- March 5, 2004 to March 24, 2004: The Selection and Interviewing Committee chose prospective candidates and interviewed them.
- March 24, 2004: Interviews with students were completed and committee members chose five students to participate in the Breast Cancer Research Training Program and matched them with BC RTP preceptors.
- March 24 to April 1, 2004: Selected students were notified of their acceptance to the program.

Task 3:        Breast Cancer Research Training Program

- May 24, 2004: Students began the Methodist Research Institute Breast Cancer Research Training Program.
- Students attended seven lectures between June 4, 2004 and July 30, 2004.
- From May 26, 2004 to August 4, 2004, students conducted breast cancer research under the guidance of their preceptors. This training included weekly meetings of all students and preceptors in the program.
- Students attended a lecture on writing research papers; preceptors guided their students in writing their research papers.
- Preceptors guided students in their preparation of oral presentations; students presented their projects informally to other students at weekly meetings.
- August 5, 2004: All students participated in rehearsal for Presentation Day.
- August 6, 2004: All students delivered oral presentations of their projects to an audience of researchers at Methodist Hospital.
- August 6, 2004: All students turned in their research papers.

Because three of the five students in the Breast Cancer Research Training Program were entering medical school in August, Presentation Day had to be moved up by one week to accommodate their orientation schedules. However, students were encouraged to continue work on their projects for the duration of the program until August 13, 2004.

Task 4.        Evaluation of the program

- August 5, 2004: Students turned in evaluation forms for the program.
- August 19, 2004: Program Coordinator wrote a final evaluation of the program.
- September 2004: Program Director and Coordinator met to review the evaluation.
- Program Director and Coordinator developed plans for improvement of the program based on the evaluation.

*Issue Raised.* During the 2004 program, one of the preceptors was indisposed with physical problems for a good part of the summer. Unfortunately, this had a considerable impact on a number of the students, as they were working in his laboratory. This was a circumstance that simply could not be anticipated. However, staff from other laboratories stepped in to help and returning students also lent a hand.

*Recommendation.* A decision was made to establish a research coordinator for the program, who could be available to guide students if and when their preceptor is not available.

*Implementation.* This was implemented for the 2005 program.

### **Key Research Accomplishments for the 2004 Program**

#### Research Accomplishments

The following paragraphs describe the projects undertaken in the 2004 Breast Cancer Research Training Program and the results of those projects.

#### **Project 1. Inhibition of breast cancer growth with *Ganoderma lucidum*.**

Brian Bock and Dr. Daniel Sliva, Cancer Research Laboratory, investigated the activity of epidermal growth factor receptor (EGFR) after treatment of MDA-MB-231 and MCF-7 breast cancer cells with *Ganoderma lucidum* (GL), an ancient Chinese medicinal mushroom. Results showed that 100 mg/kg was effective in inhibiting tumor growth compared to control. Results were inconclusive at 500 mg/kg because of the death of some of the mice. However, it can be concluded that GL can be a potential inhibitor of tumor growth in highly invasive breast cancer cells.

#### **Project 2. Effect of kinase inhibitors on ERK activity in breast cancer cells.**

Jennifer Griffith worked with Dr. A. Thomas Kovala of the Experimental Cell Research Laboratory to test the ability of various compounds to directly inhibit ERK activity in the mitogen-activated protein kinase (MAPK) pathway and to sensitize breast cancer cells to apoptosis. AG1296 was found to inhibit ERK phosphatase activity, while ERK kinase activity was not affected. SU1498 was found to increase apoptosis in both highly aggressive breast cancer cell lines, though it is not uniformly effective. AG1007 did not induce an increase in apoptosis in any of the cell lines. Thus, SU1498 remains the kinase inhibitor with the greatest potential in cancer treatment.

#### **Project 3. Omega-3 fatty acids, apoptosis, and membrane organization.**

Laura Sech worked with Drs. Gary Zaloga and Rafat Siddiqui, Cellular Biochemistry Research Laboratory, investigating the role of omega-3 fatty acids in inducing apoptosis in MDA-MB-231 breast cancer cells. In the present study, it was hypothesized that various fatty acids would be localized into nonraft domains—as opposed to raft domains. This was determined using the biochemical detergent extraction method in breast cancer cells. In accordance with the hypothesis, EPA, retinol, and DHA were localized predominantly in the nonraft regions.

**Project 4. Effects of C-reactive protein (CRP) and CRP peptide (RS 83277) on activation of NF- $\kappa$ B and upregulation of uPA and uPAR.**

C-reactive protein (CRP) is an acute-phase protein released in the liver in response to inflammation. Chelimo Yego and Dr. Carlos Labarrere, Experimental Pathology Laboratory, hypothesized that the CRP peptide RS 83277 increases MDA-MD-231 breast cancer cell growth by activating nuclear factor-kappa B (NF- $\kappa$ B) and subsequently upregulating urokinase plasminogen activator (uPA) and its receptor uPAR. The investigators concluded that CRP and CRP peptide RS 83277 may promote the growth of breast cancer cells as secretion of uPA is increased. Activation of NF- $\kappa$ B will consequently promote the upregulation of uPA and uPAR, resulting in increased migration, adhesion, and invasion of cells.

**Project 5. Role of the calpain proteolytic pathway in breast cancer-induced cachexia.**

In a study continuing from the 2003 Summer Student Research Program, Heidi Yount and Drs. Gary Zaloga and Rafat Siddiqui examined whether two omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), inhibit the epidermal growth factor (EGF) receptor, and hence, activation of the calpain pathway. Results showed that DHA decreased proteolysis of skeletal muscle cells treated with media alone and media from MDA-MB-231 cells containing proteolysis-inducing factor. Gelatin zymography studies were not complete at the time, but the results were expected to show a decrease in the amount of calpain protease activity in the cells treated with DHA, supporting the conclusion that DHA inhibits proteolysis by modulating the calpain proteases. Results for the effect on activation of the EGF receptor were inconclusive.

**Reportable Outcomes for the 2004 Program** (Students' names are in bold.)

**Press Release/News Item**

Preceptor: Rafat Siddiqui

"Dietary fish oil curbs breast cancer progression in animal study." Reuters Health, last updated 2005-11-24.

Anne Marie Tiernon, "Fish oil shows promise in treating cancer." WTHR Channel 13 Eyewitness Health, <http://www.wthr.com/Global/story.asp?s=4178806>. Accessed 4/13/2006.

**Manuscripts**

Preceptors: Rafat Siddiqui and Gary Zaloga

Wu M, Harvey KA, Welch ZR, **Sech L**, Jackson K, Stillwell W, Zaloga GP, Siddiqui RA. Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway. *Int J Cancer*. 2005;117:340-348.

**Presentations**

Preceptors: Rafat Siddiqui and Gary Zaloga

Siddiqui RA, Wu M, Harvey KA, Welch ZR, **Sech L**, Jackson K, Stillwell W, Zaloga GP. Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway. *FASEB J*. 2004;19:A1693 (oral presentation at Experimental Biology Meeting). \*Student's name left out in journal.

Siddiqui RA, Wu M, Ruzmetov N, Harvey KA, Welch ZR, **Sech L**, Jackson K, Zaloga GP, Stillwell W. Neutral sphingomyelinase mediates inhibitory effects of omega-3 polyunsaturation on breast cancer development. American Association for Cancer Research International Research Conference on Food, Nutrition, and Cancer, July 11-15, 2005, Washington, DC.

### **Conclusions**

We recruited twenty-four applicants, interviewed seven candidates, and selected five individuals for the 2004 Breast Cancer Research Training Program at the Methodist Research Institute (MRI). Two students returned to participate in the 2004 program after being in the 2003 program. One student participated in the general Summer Student Research Program in 2003 and was accepted into the BCRT in 2004. Trainees attended lectures dealing with research design, statistics, ethics, and research reporting. Trainees developed and worked on individual research projects under the supervision of a mentor. Each trainee significantly contributed to larger breast cancer research projects. Three of the five projects dealt with dietary modulation of breast cancer cell growth and progression and showed that both *Ganoderma lucidum* (a Chinese mushroom) and omega-3 polyunsaturated fatty acids have anticancer effects. Another project evaluated the effects of C-reactive protein (a proinflammatory peptide) upon signaling pathways involved in cancer cell growth. CRP was found to promote signaling associated with tumor progression. A final study evaluated the effect of ERK inhibition upon cancer cell apoptosis. A specific ERK inhibitor promoted apoptosis. The knowledge obtained from these studies contributed significantly to ongoing studies at MRI and contributed to our knowledge of dietary effects upon breast cancer progression. Three of our students entered medical school following the program.

### **2005 Breast Cancer Research Training Program**

#### **Body**

Planning for the Breast Cancer Research Training Program began in October 2004 with development of application materials and student recruitment. The application due date was February 16, 2005, and students were offered positions in late March to early April. Students began the program on May 23<sup>rd</sup> and the program ended August 12<sup>th</sup> with Presentation Day.

Thirty-six students were recruited to the 2005 Breast Cancer Research Training Program. Eight of the applicants to the program were from underrepresented minorities. We interviewed seven candidates for four positions in the program (one student from the general Summer Student Research Program in 2004 was accepted into the 2005 Breast Cancer Research Training Program).

The following list presents the students who were ultimately chosen, the college or university attended and class status at the time of application, and their BCRT program preceptor(s):

Student	Class and Institution	Preceptor
Lauren Cottee (minority)	Sophomore, Indiana University	Carlos A. Labarrere
Brian Grieb	Sophomore, Centre College	Daniel Sliva
Diana Herrera (minority)	Junior, Purdue University	Rafat A. Siddiqui
Jun Kawasaki	Junior, Grinnell College	Daniel Sliva
Neal Patel	Senior, Indiana University	Rafat A. Siddiqui

One student was accepted into medical school during the course of the summer. Another student has been accepted into a graduate cancer research program at Harvard University.

### 2005 Training Accomplishments According to the Statement of Work

#### Task 1: Recruitment of undergraduate students interested in breast cancer research training

- October 2004: Program application updated and information materials prepared and uploaded onto the website
- October through November 2004: Information packages with application materials to students, colleges/universities, and community leaders mailed. Application packages and brochures were mailed out in response to requests for applications.
- December 2004: Follow-up phone calls were made and brochures were sent out to promote the Breast Cancer Research Training Program at Methodist Research Institute.

#### Task 2. Selection of students to participate in the Breast Cancer Research Training Program

- February 16, 2005: All applications received by February 16, 2005 or with a postmark of February 16, 2005 were considered for the program.
- March 18, 2005 to March 28, 2005: The Selection and Interviewing Committee chose prospective candidates and interviewed them.
- March 28, 2004: Interviews with students were completed and committee members chose five students to participate in the Breast Cancer Research Training Program and matched them with BC RTP preceptors.
- March 28 to April 7, 2005: Selected students were notified of their acceptance to the program.

#### Task 3: Breast Cancer Research Training Program

- May 23, 2005: Students began the Methodist Research Institute Breast Cancer Research Training Program.
- Students attended seven lectures between June 7, 2005 and July 29, 2005.
- From May 25, 2005 to August 10, 2005, students conducted breast cancer research under the guidance of their preceptors. This training included weekly meetings of all students and preceptors in the program.

- Students attended a lecture on writing research papers; preceptors guided their students in writing their research papers.
- Preceptors guided students in their preparation of oral presentations; students presented their projects informally to other students at weekly meetings.
- August 11, 2005: All students participated in rehearsal for Presentation Day.
- August 12, 2005: All students delivered oral presentations of their projects to an audience of researchers at Methodist Hospital.
- August 12, 2005: All students turned in their research papers.

#### Task 4.           Evaluation of the program

- August 11, 2005: Students turned in evaluation forms for the program.
- August 23, 2005: Program Coordinator wrote a final evaluation of the program.
- September 2005: Program Director and Coordinator met to review the evaluation.
- Program Director and Coordinator developed plans for improvement of the program based on the evaluation.

*Issue Raised.* A day was set aside during the 2005 program for Career Day—an opportunity to introduce students to aspects of a typical biomedical researcher career. Presentations were offered on radiation and biosafety, clinical trials, grant funding, and the MD/PhD Program at Indiana University School of Medicine. Response from the students, however, was not enthusiastic.

*Recommendation.* While certain presentations were successful and should be retained in 2006, the Career Day as a whole should be eliminated.

*Implementation.* Career Day will not be offered in the 2006 program.

Overall, students seemed to value most the experience of working one-on-one with a biomedical researcher.

### **Key Research Accomplishments for the 2005 Program**

#### Research Accomplishments

The following paragraphs describe the projects undertaken in the 2005 Breast Cancer Research Training Program and the results of those projects.

**Project 1. Effects of omega-3 fatty acids and polyphenols on breast cancer cell growth.** Diana Herrera worked with Dr. Rafat Siddiqui of the Cellular Biochemistry Laboratory on a project investigating the hypothesis that docosahexaenoic acid (DHA) synergizes the antioxidant effects of polyphenols to induce apoptosis in breast cancer cells. The effects of DHA on polyphenol-induced apoptosis in breast cancer cells did not appear to be mediated through its oxidation/antioxidation effects.

**Project 2. Investigation into anticancer properties of polyphenols and omega-3 fatty acids in breast cancer cells.**

Neal Patel and Dr. Rafat Siddiqui investigated the mechanisms by which omega-3 fatty acids and polyphenols exert their anticancer effects in breast cancer—in particular, their effects on protein phosphorylation. They concluded that dietary polyphenols and omega-3 fatty acids may inhibit breast cancer growth by activating protein phosphatases, which activate a kinase downstream, causing phosphorylation of Thr 180/Tyr 182 residues on p38 kinase, a regulatory protein involved in cell apoptosis.

**Project 3. Effect of CRP RS 83277 peptide against mammary adenocarcinoma (EMT6) in BALB-C mice.**

Lauren Cottee worked together with Dr. Carlos Labarrere and Dr. Miguel Ortiz of the Experimental Pathology Laboratory investigating the hypothesis that treatment of BALB-C mice with mammary adenocarcinoma with the C-reactive protein peptide RS 83277 will slow or stop the progression of the primary tumor and will stimulate tumor rejection in the T-lymphocyte immune response. The investigation found that a relative increase in CD8+ cells compared to CD4+ cells in CRP RS 83277 peptide-treated mice could facilitate the killing of tumor cells, which is manifested by increased necrotic tissue.

**Project 4. Inhibitory effects of *Phellinus linteus* on highly invasive breast cancer cells.**

Jun Kawasaki and Dr. Daniel Sliva of the Cancer Research Laboratory considered the hypothesis that *Phellinus linteus*, a Japanese medicinal mushroom, inhibits cell proliferation, adhesion, migration, and invasion of highly invasive MDA-MB-231 breast cancer cells by downregulating the Akt and MAPK activities, resulting in the inhibition of AP-1 and NF- $\kappa$ B transcription factors. *Phellinus linteus* displayed its anticancer effects on invasive breast cancer cells by inhibiting Akt kinase.

**Project 5. Effect of *Ganoderma lucidum* triterpenes on highly invasive MDA-MD-231 breast cancer cells.**

Brian Grieb worked with Dr. Daniel Sliva to test five purified triterpenes to determine whether they exhibit anticancer properties independently of other components of *Ganoderma lucidum*. They concluded that, while the isolated triterpenes did not behave identically to the entire *G. lucidum* mushroom, they exhibited anticancer properties and probably contribute to the overall effect of the mushroom.

**Reportable Outcomes for the 2005 Program (Students' names are in bold)**

**Manuscripts**

None.

**Presentations**

Preceptor: Rafat Siddiqui

Siddiqui RA, Harvey KA, **Herrera D**, **Patel N**, Parnavitana C, Stillwell W, Zaloga G. Docosahexaenoic acid and polyphenols synergistically induce apoptosis in breast cancer cells by activating protein phosphatases. Experimental Biology Meeting, San Francisco, CA. April 1-5, 2006.

Siddiqui RA, Ruzmetov N, Harvey KA, Zerouga M, Terry C, Patel N, Stillwell W, Zaloga GP. Omega-3 fatty acids inhibit protein kinase A and calcium calmodulin kinase II activities and improve survival following myocardial infarction. 2<sup>nd</sup> Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences—Targeting Heart Failure: New Science, New Tools, New Strategies, Keystone, CO. July 24-27, 2005.

### Conclusions

Thirty-five students applied to the 2005 Breast Cancer Research Training Program. We selected four new students to participate in the program. The fifth student returned to us after participating in the 2004 general Summer Student Research Program. Trainees attended lectures, weekly meetings, and a Career Day. Ninety percent of their time in the program was spent developing and working on their individual research projects with their preceptors. These projects contributed greatly to larger, ongoing breast cancer research projects at Methodist Research Institute. Two projects focused on the anticancer effects of omega-3 fatty acids and polyphenols in breast cancer cells. Two other projects looked at the Asian medicinal mushrooms, *Ganoderma lucidum* and *Phellinus linteus*. Triterpenes in *G. lucidum* were tested for the mechanisms of their anticancer properties, independently of the mushroom as a whole. The mechanism by which *P. linteus* inhibits cell proliferation, adhesion, migration, and invasion in highly invasive breast cancer was also examined. A fifth student considered the effectiveness of a C-reactive protein peptide, RS 83277, in slowing or stopping progression of adenocarcinoma in BALB-C mice. One student has entered medical school; another student has been accepted into a cancer research graduate program at Harvard University.

We are proud of the students who have participated in the Army MRMC-sponsored Breast Cancer Research Training Program from 2003 through 2005 and are proud to have contributed to their formation in biomedical research training in breast cancer.

## APPENDICES

### **Reportable Outcomes for 2003 Program**

#### **Manuscripts:**

Preceptors: Rafat Siddiqui and Gary Zaloga

Siddiqui R, Shaikh S, **Sech L**, **Yount H**, Stillwell W, Zaloga G. Omega-3 fatty acids: health benefits and cellular mechanisms of action. *Minirev Med Chem*. 2004;4:859-871.

#### **Presentations:**

Preceptor: Rafat Siddiqui

**Yount H**, Siddiqui R. Role of omega-3 fatty acids in the prevention of cancer-induced muscle proteolysis. 227th ACS National Meeting, Anaheim, CA. March 27 - April 1, 2004, Abstract #CHED242.

### **Reportable Outcomes for 2004 Program**

#### **Press Release/News Item**

Preceptor: Rafat Siddiqui

“Dietary fish oil curbs breast cancer progression in animal study.” Reuters Health, last updated 2005-11-24.

Anne Marie Tiernon, “Fish oil shows promise in treating cancer.” WTHR Channel 13 Eyewitness Health, <http://www.wthr.com/Global/story.asp?s=4178806>. Accessed 4/13/2006.

#### **Manuscripts**

Preceptors: Rafat Siddiqui and Gary Zaloga

Wu M, Harvey KA, Welch ZR, **Sech L**, Jackson K, Stillwell W, Zaloga GP, Siddiqui RA. Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway. *Int J Cancer*. 2005;117:340-348.

#### **Presentations**

Preceptors: Rafat Siddiqui and Gary Zaloga

Siddiqui RA, Wu M, Harvey KA, Welch ZR, **Sech L**, Jackson K, Stillwell W, Zaloga GP. Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway. *FASEB J*. 2005;19:A1693 (oral presentation at Experimental Biology Meeting). \*Student’s name left out in journal.

Siddiqui RA, Wu M, Ruzmetov N, Harvey KA, Welch ZR, **Sech L**, Jackson K, Zaloga GP, Stillwell W. Neutral sphingomyelinase mediates inhibitory effects of omega-3 polyunsaturation on breast cancer development. American Association for Cancer Research International Research Conference on Food, Nutrition, and Cancer, July 11-15, 2005, Washington, DC.

### **Reportable Outcomes for 2005 Program**

#### **Presentations**

Preceptor: Rafat Siddiqui

Siddiqui RA, Harvey KA, **Herrera D**, **Patel N**, Parnavitana C, Stillwell W, Zaloga G. Docosahexaenoic acid and polyphenols synergistically induce apoptosis in breast cancer cells by activating protein phosphatases. Experimental Biology Meeting, San Francisco, CA. April 1-5, 2006.

Siddiqui RA, Ruzmetov N, Harvey KA, Zerouga M, Terry C, **Patel N**, Stillwell W, Zaloga GP. Omega-3 fatty acids inhibit protein kinase A and calcium calmodulin kinase II activities and

improve survival following myocardial infarction. 2<sup>nd</sup> Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences—Targeting Heart Failure: New Science, New Tools, New Strategies, Keystone, CO. July 24-27, 2005.

# Omega 3- Fatty Acids: Health Benefits and Cellular Mechanisms of Action

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**Abstract:** Epidemiological evidence has established that ingestion of long chain, polyunsaturated omega-3 fatty acids, ( $\omega$ -3 PUFAs) abundant in fish oils, have profound effects on many human disorders and diseases including cardiovascular disease and cancer. Here we briefly review the dietary recommendations and the food sources that are naturally enriched in these fatty acids. There are also a number of products including eggs, bread, and cereals available to supplement  $\omega$ -3 fatty acid dietary intake. Some of these supplements are proposed to aid different pathological conditions. While the beneficial effects of omega-3 fatty acids can no longer be doubted, their molecular mechanism of action remains elusive. Without question, the action of omega-3 fatty acids is complex and involves a number of integrated signaling pathways. This review focuses on one of the possible cellular mechanisms by which the  $\omega$ -3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), may function. Studies with cancer cells suggest that DHA induces cell cycle arrest and apoptosis by activating protein phosphatases leading to dephosphorylation of retinoblastoma protein (pRB). Protein phosphatases are also involved with a protein Bcl2, which regulates the release of cytochrome c from mitochondria and eventually, activation of the apoptotic enzyme caspase 3.

**Keywords:** Omega 3- fatty acids, Cancer, Dietary intake, Food supplements.

## INTRODUCTION

It is now evident that "all fats are not created equal". Some fats such as cholesterol saturated fats and polyunsaturated long chain omega-6 fatty acids taken in excess are considered "bad" for human health while the class of long chain polyunsaturated omega-3 fatty acids ( $\omega$ -3 PUFAs) are beneficial. Table 1 presents a partial list of human afflictions that have been alleviated by  $\omega$ -3 PUFAs. It is unclear how this class of very simple molecules can affect so many seemingly unrelated diseases. The reasons behind the beneficial properties of the omega-3 fatty acids are the subject of considerable interest and intense investigation.

## EPIDEMIOLOGY STUDIES

The first clue that  $\omega$ -3 PUFAs may exert beneficial effects on human health came from epidemiology studies on populations in which fish was a major component of the diet. The favorable health effects of  $\omega$ -3 PUFAs on the cardiovascular system was initially recognized by Dyerberg *et al.* in the 1970s [1]. These researchers observed that Greenland Eskimos, who consumed a diet rich in  $\omega$ -3s, had a low rate of cardiovascular disease as measured by a number of factors. Similar observations were also made for a Japanese fishing village that consumed an average of 250g of fish daily compared to a Japanese farming village that only averaged 90g of fish daily [2]. There are now many studies that have found an inverse association between fish oil consumption and risk of coronary heart disease (CHD) or

sudden cardiac death in the general population [3-7]. In addition to the beneficial cardiovascular effects, use of fish oil was also reported to have anticancer properties. An epidemiology study of South African West Coast fisherman reported that despite smoking; high sodium intake; low consumption of fiber, fruits, and vegetables; absence of vitamin supplementation; and low levels of dietary micronutrients compared to urban whites, the fisherman had a lower incidence of colorectal cancer [8]. This was attributed to the protective effects of fish oil in their diets [8]. Similarly, a population-based case-control epidemiology study in Norway demonstrated an inverse relationship between serum  $\omega$ -3 PUFA concentrations and thyroid cancer [9]. Cross-national studies have shown an inverse relationship between fish consumption and incidences of and mortality rates from prostate [10, 11] and breast cancer [12-16]. Furthermore, a series of case-controlled studies in Italy and Switzerland suggest that  $\omega$ -3 PUFAs decrease the risk of several cancers, including oral and pharyngeal, esophageal, colon, breast, and ovarian cancers [17].

## OMEGA-3 FATTY ACIDS AND THE DIET

Soon after the original epidemiology studies on fish oil diets became appreciated, it was evident that the "Western diet", rich in saturated fats, was partially responsible for the high incidence of cancer and heart disease associated with modern societies. An emphasis was then placed on substituting animal fats with unsaturated vegetable oils from corn, sunflower seeds, safflower seeds, cottonseed, and soybeans. Since these oils are rich in  $\omega$ -6 fatty acids, there has been an associated increase in the  $\omega$ -6/ $\omega$ -3 dietary lipid ratio in Western societies. The  $\omega$ -3 and  $\omega$ -6 families of PUFAs function differently because of the location of the last double bond in the 3<sup>rd</sup> (omega-3) or 6<sup>th</sup> (omega-6)

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**Table 1. Reported Beneficial Effects of  $\omega$ -3 PUFAs in Various Diseases**

Disease/Disorders	Reference
ADHD	[135]
Aggression	[136]
Alcoholism	[137]
Arthritis	[138]
Asthma	[139,140]
Bipolar Disorder	[141]
Blindness	[142]
Cancer	[9,17,88,143-149]
Crohn's Disease	[150]
Cystic Fibrosis	[151]
Depression	[152]
Dermatitis	[153]
Dyslexia	[154]
Heart Disease	[155,156]
Hypersensitivity	[157]
Kidney Disease	[158]
Lupus Erythematosus	[159]
Malaria	[160]
Migraine Headaches	[161]
Multiple Sclerosis	[162]
Neurovisual Developmental Disorders	[163]
Nephropathy	[164]
Peroxisome Biogenesis Disorder	[165]
Phenylketonuria	[166]
Psoriasis	[167]
Respiratory Diseases	[168]
Schizophrenia	[169]
Suicide	[152]
Ulcerative Colitis	[26,27]

positions from the methyl terminal of the aliphatic carbon chain Fig. (1). In a typical Western diet, the ratio of  $\omega$ -6 to  $\omega$ -3 fatty acids now ranges from approximately 20-30:1 instead of the range of 1-2:1, which is believed to have been present in the diets of prehistoric populations that survived on fresh fruits, vegetables, fish and animals [18]. A similar low ratio of  $\omega$ -6/ $\omega$ -3 dietary lipids has been reported for modern populations subsisting on a fish-based diet [1, 19]. Corresponding to this dramatic change in PUFA dietary ratio is an increased risk of cardiovascular, cancer, and other diseases among Western populations compared to

populations that lived before the Industrial Revolution and those currently living on diets rich in fish oils [8, 9, 20-22]. The beneficial effects of fish oils are mostly attributed to their content of the  $\omega$ -3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

The 18-carbon  $\omega$ -3 PUFA,  $\alpha$ -linolenic acid, found in green leafy vegetables, flaxseed, rapeseed, and walnuts, can be desaturated and elongated in the human body to EPA and then to DHA. Therefore  $\alpha$ -linolenic acid may have similar beneficial effects on health to the longer chain PUFAs. The  $\omega$ -3 and  $\omega$ -6 fatty acid families are important for human nutrition. The precursors of these fatty acids, 18-carbon linoleic ( $\omega$ -6) and  $\alpha$ -linolenic ( $\omega$ -3), cannot be produced in the body and are therefore "essential" to the diet. Linoleic acid and linolenic acid are converted to longer chain  $\omega$ -6 and  $\omega$ -3 fatty acids by various cycles of desaturation and elongation as presented in Fig. (2).

The most common food sources of the long chain PUFA  $\omega$ -3 fatty acids are cold-water fatty fish, including mackerel, salmon, herring, trout, sardines, and tuna (Table 2). Eggs and meat also contain small amounts of  $\omega$ -3 fatty acids (Table 3). Increased intake of  $\omega$ -3 PUFA can also occur through consumption of dietary  $\alpha$ -linolenic acid, which can be metabolically converted, to EPA and DHA. Alpha-linolenic acid is a common component of flax seeds and canola oil. Flax seeds contain approximately 24% while canola oil contains approximately 11%  $\alpha$ -linolenic acid. Canola oil is readily available in many foods such as bread and cereals, while energy bars often contain flax seed oils.

Because of the health benefits that  $\omega$ -3 fatty acids provide, there is a need to set a Recommended Daily Allowance guideline for  $\omega$ -3 fatty acids. As of yet, the United States Food and Drug Administration (FDA) has not made such a recommendation. In the typical Western diet, the average  $\omega$ -3 fatty acid consumption is less than 0.1 grams/day. This is a very small amount considering that health authorities in Canada\*, the United Kingdom†, and Australia‡ have made recommendations of 1-2 g  $\omega$ -3 PUFAs/day. In 1999, as part of an effort to evaluate the importance of  $\omega$ -3 PUFAs, a workshop was held at the National Institute of Health (Bethesda, Maryland, USA) to determine the recommended dietary intakes of  $\omega$ -6 and  $\omega$ -3 fatty acids. This workshop established an amount representing the Adequate Intake for Adults and Infants. For a 2000 kcal diet, the recommended intake of  $\omega$ -3 fatty acids for adults was 0.65 grams/day, with a minimum intake of  $\omega$ -3 fatty acids being 0.22 grams/day. The Adequate Intake for Infant Formula was set at approximately 2% of lipid intake [23]. More recently the American Heart Association recommended 1 gm/day of  $\omega$ -3 PUFA for adults for the prevention of cardiovascular disease [24].

### OMEGA-3 FATTY ACID SUPPLEMENTATION

Despite the fact that the Food and Drug Administration has yet to recommend precise dietary intake of  $\omega$ -3 and  $\omega$ -6

\* Nutrition Recommendations. In S.R. Committee, Ed.; Minister of National Health and Welfare: Ottawa, 1990; pp. H49.

† Unsaturated fatty acids-nutritional and physiological significance: the report of the British Nutrition Foundation's Task Force. In C.a. Hall, Ed.; The British Nutrition Foundation: London, 1992.

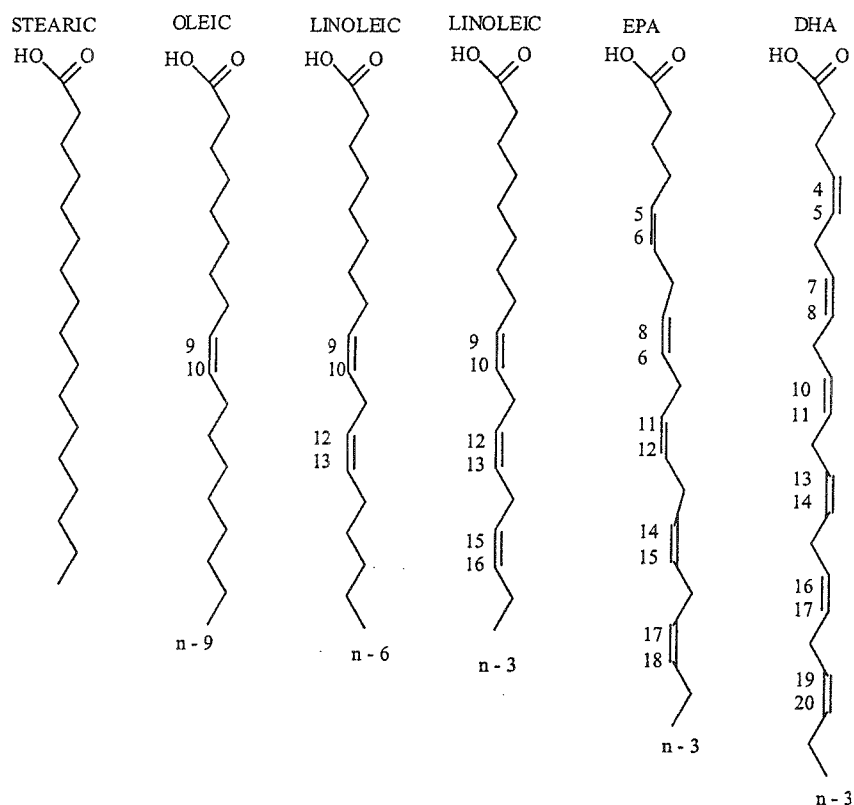


Fig. (1). Some biologically important fatty acids

Fatty acids are classified in saturated,  $\omega$ -3,  $\omega$ -6, or  $\omega$ -9 families based on the position of the last double bond at the 3<sup>rd</sup>, 6<sup>th</sup>, or 9<sup>th</sup> position from the methyl terminal of the aliphatic carbon chain.

<sup>†</sup> Report of the NHMRC working party: the role of polyunsaturated fats in the Australian diet. 1992.

PUFAs, there is little doubt that fatty acids can have a profound effect on human health. This realization has paved the way for increased marketing of  $\omega$ -3 supplements and patents for future drug development.

There are hundreds of web sites advertising the availability of  $\omega$ -3 products as well as countless formulas rich in  $\omega$ -3 that have been patented. Most of the patents contain different mixtures of  $\omega$ -3 and  $\omega$ -6 fatty acids and are suggested for the treatment of a myriad of disorders (Table 4). Although it is beyond the scope of this review to thoroughly discuss the countless applications of the patented formulas, a few representative examples follow. Their uses range from enhancing the weakened immune system of trauma patients to combating the effects of aging caused by cigarette smoke. Some formulations comprise a liquid drink while others may be administered enterally to ICU patients who have depressed absorption capacity. Other patents include a method for restoring gut integrity as well as a formulation to combat the negative symptoms found in postmenopausal women.

Perhaps the most abundant area for development of  $\omega$ -3 patents has been in the treatment of inflammatory diseases. Compositions containing  $\omega$ -3 esters have been developed and clinically proven to treat psoriasis and phlebitis [25] as well as ulcerative colitis [26, 27]. For the treatment of chronic inflammation as well as liver disorders, a combination of various  $\omega$ -3 fatty acids has been developed

in the form of medium-length triglycerides. The administration of  $\omega$ -3 fatty acids as medium-length triglycerides speeds clearance of the lipid emulsions from the blood and therefore provides certain advantages. Enhanced blood clearance of these lipids results from stimulation of specific tissue uptake and inhibition of the synthesis of endogenous triglycerides. Thus, the uptake of  $\omega$ -3s as medium-length triglycerides may also contribute to an overall reduction of blood triglycerides [28]. Furthermore, accompanying  $\omega$ -3 PUFAs with medium-length triglycerides protects them from rapid oxidation, and this combination alone has a protective effect on the liver [29, 30].

Because of the increased research involving the health benefits of  $\omega$ -3 fatty acids, several functional foods have been designed to enhance  $\omega$ -3 PUFA intake (Table 5). Two functional foods have been designed for very specific purposes. For infants, Mead Johnson Nutritionals, (Bristol-Myers Pharmaceuticals, Evansville, IN) makes Enfamil Lipil<sup>™</sup> brand baby formula, with levels of  $\omega$ -3 PUFA comparable to breast milk and significantly higher than other commercial formulas. DHA in infants is important because it is essential for proper brain and eye development. For cancer patients, Ross Pharmaceutical (Abbott Laboratories, Columbus, OH) makes Prosure, a nutrition and energy beverage containing DHA designed to help people reverse tumor-induced weight loss. Omega-3 PUFAs intake may be increased by the use of  $\omega$ -3 PUFAs enriched eggs,

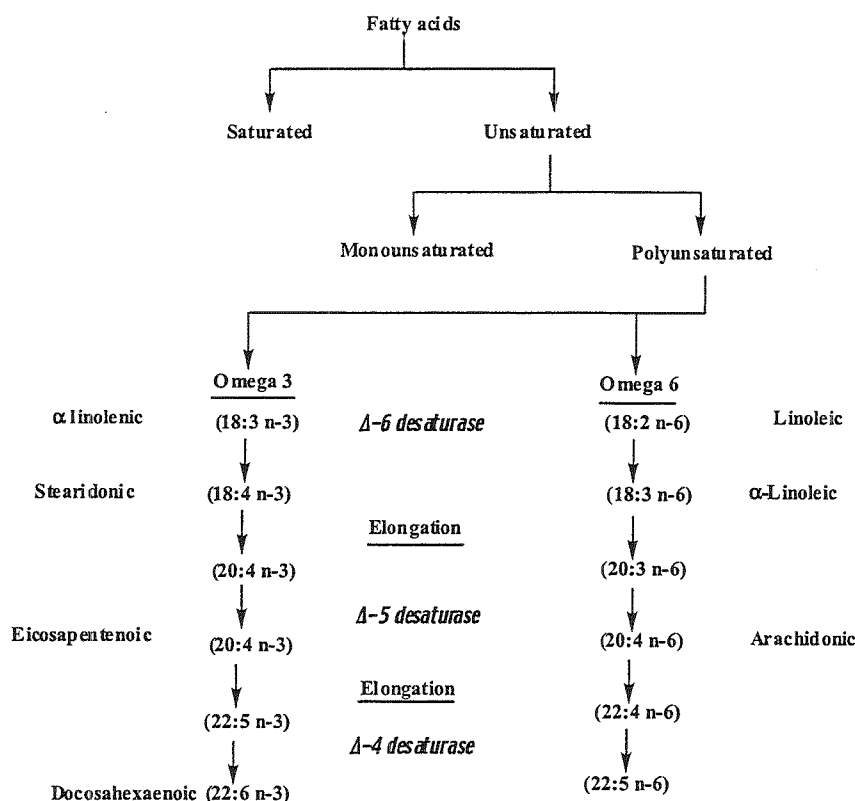


Fig. (2). Metabolic pathway of omega 6 and omega 3 fatty acid synthesis

Fatty acids are classified as saturated or unsaturated fatty acids depending on the presence of double bonds. The unsaturated fatty acids are further divided into monounsaturated or polyunsaturated fatty acids. The polyunsaturated fatty acids are either  $\omega$ -3 or  $\omega$ -6 fatty acids.  $\alpha$ -linolenic acid and linoleic acid are the precursors of  $\omega$ -3 and  $\omega$ -6 fatty acids, respectively, and are converted to different long chain polyunsaturated fatty acid by sequential desaturation and elongation.

manufactured by many companies across Canada and the United States.

We analyzed seven different brands of  $\omega$ -3 Fish oils (Sundown Flax Seed Oil, Sundown Cod Liver Oil, Sundown Fish Oil, Puritan's Pride Super EPA, Nature Made Fish Oil, Member's Mark Fish Oil, and Sigma-Aldrich Fish Oil) for their chemical composition (Table 6). It is clear from the labels of ingredients that different  $\omega$ -3 supplements contain different amounts of  $\omega$ -3 PUFA ranging from 160 to 550 mg/g of oil. Our gas chromatographic analysis data further indicate some differences in quantities of  $\omega$ -3 PUFA in the capsules versus the amount that is marketed by the manufacturers. These discrepancies could be due to the fact that the oils are extracted from fish, which may be caught from different environmental conditions (cold water versus warm water) and in different seasons. These  $\omega$ -3 PUFA supplements also have different levels of lipid peroxidation (data not shown) because each supplement may have been processed and stored differently as well as supplemented with different antioxidants. Furthermore, our data also indicate that fish oil supplements contain a significant quantity of other unsaturated fatty acids that nutrition labels neglect to reveal. While the appearance of other unsaturated fatty acids may have no effect on the ability of  $\omega$ -3 PUFAs to function properly, the presence of other unsaturated fatty acids may have adverse biological effects. It is therefore important that

these fatty acid supplements be evaluated and standardized against biological activities.

Because of the reputed ability of  $\omega$ -3 fatty acids to support such a wide variety of health benefits, they could be considered as "wonder drugs". However, FDA does not classify these nutritional compounds as drugs and the FDA does not officially recognize them as treatments for the diseases. Despite this fact, the patented formulas and marketed nutritional supplements that contain  $\omega$ -3 lipids will most certainly continue to be developed.

#### A POSSIBLE MODE OF ACTION FOR OMEGA-3 PUFA

Although epidemiological and nutritional evidence strongly suggest that  $\omega$ -3 PUFAs have an influence on various disease states, the mechanism by which  $\omega$ -3 PUFAs function remains unclear. Whatever omega-3 fatty acids mode of action is, it must be fundamental and commonly shared by a wide variety of tissues. Several non-exclusive hypotheses regarding the mode of action of  $\omega$ -3 PUFAs have been proposed. It has been suggested that  $\omega$ -3 PUFAs may affect numerous membrane properties (e.g., permeability [31], "fluidity" [32], lipid packing [33], fusion [32], deformability [34] etc.); the activity of specific proteins (e.g., protein kinase C [35], rhodopsin [33], (Na<sup>+</sup>, K<sup>+</sup>)-

Table 2.  $\omega$ -3 Fatty Acid (DHA and EPA) Content in Seafood<sup>1</sup>

Type	Amount of DHA (g/3oz portion)	Amount of EPA (g/3oz portion)	Amount Required to Provide $\approx$ 1 g of DHA and EPA
Catfish			
Farmed	0.116	0.085	15 oz
Wild	0.109	0.042	20oz
Clams	0.124	0.177	10 oz
Cod			
Atlantic	0.131	0.003	23 oz
Pacific	0.147	0.088	13 oz
Crab, Alaskan King	0.100	0.251	9 oz
Flounder/Sole	0.219	0.207	7 oz
Halibut	0.318	0.077	8 oz
Lobster	0.026	0.045	42 oz
Oyster			
Eastern	0.496	0.456	3 oz
Farmed	0.179	0.195	8 oz
Pacific	0.425	0.745	3 oz
Salmon			
Atlantic Farmed	1.238	0.587	2 oz
Atlantic Wild	1.215	0.349	2 oz
Pink Canned	0.685	0.718	2 oz
Scallops	0.092	0.076	18 oz
Shrimp	0.122	0.145	11 oz
Trout, Rainbow			
Farmed	0.697	0.284	3 oz
Wild	0.442	0.398	4 oz
Tuna			
Canned, light	0.190	0.040	13 oz
Fresh	0.970	0.309	2 oz
White	0.535	0.198	4 oz

<sup>1</sup>USDA Nutrient Data Laboratory. <http://www.nalusda.gov/fnic/foodcomp/>. Accessed August 5, 2003.

ATPase [36], and Na<sup>+</sup> channel [37]); lipid microdomain formation; [38-40] eicosanoid biosynthesis; [41] gene expression; [42] and formation of potent lipid peroxidation products [43]. It is likely that a combination of several of

these effects are responsible for the beneficial health properties of  $\omega$ -3 PUFAs. Here we will describe one possible molecular action of omega-3 fatty acids, their role in programmed cell death (apoptosis).

Table 3. Foods Naturally Containing  $\omega$ -3 PUFAs<sup>2</sup>

Food	Amount of $\omega$ -3 PUFA (mg)
1 large hard-boiled egg	19
2 pieces fried chicken	37
3 oz tuna salad	47
12 large steamed shrimp	96
1 cup chicken livers	112
3 oz steamed crab	196
3 oz smoked salmon	227
3 oz beef liver	246
3 oz white tuna	535
3 oz salmon fillet	638

<sup>2</sup>U.S. Department of Agriculture, Agriculture Research Service, 1999. USDA Nutrient Database for Standard Reference, Release 13.

Omega-3 PUFAs induce apoptosis in cancer cells, whereas they protect neuronal, retinal, and cardiac cells against apoptosis. It is beyond the scope of this review to discuss the effect of  $\omega$ -3s in every single health condition. Therefore, this review article will focus primarily on the role of  $\omega$ -3 PUFAs in effecting signal transduction processes leading to apoptosis in various cancers.

Several reports have demonstrated that  $\omega$ -3 PUFAs exert their anticancer effects on various cancer cell lines [44-47]. Dietary supplementation with  $\omega$ -3 PUFAs (as a pure agent or in fish oil) increased apoptotic cell death in normal rat colonic cells [44-47], in transplantable rat Morris hepatocarcinoma 3924A [48] and Walker 256 carcinosarcoma [49]. Omega-3 PUFAs suppress the progression of human breast MDA-MB-231 [50, 51], MDA-MB-435 [52, 53], and KPL-1 cancer cells [54] in athymic nude mice. They increase survival time for dogs with lymphoma [55]; and reduce the

Table 4. Therapeutic Use of  $\omega$ -3 PUFAs

Patent	Description	Inventor	Patent #
Glutamine-rich composition for immune system	For the purpose of treating patients whose immune systems have been weakened because of disease or trauma; the major ingredient is glutamine, which is accompanied by $\omega$ -3 and $\omega$ -6 PUFAs, arginine, and RNA.	Steven M. Ostrom	199861/10 USA
Formulation for menopausal women	For the purpose of providing nutritional supplementation for postmenopausal/menopausal women as well as relieving associated symptoms. The supplementation consists of various compounds including linoleic acid, linolenic acid, DHA, $\omega$ -2 fatty acids, and other $\omega$ -3 fatty acids.	Saul R. Levinson et al.	131236/10 USA
Composition for increase in $\omega$ -3 of human cell membranes	Proposes to increase the amount of $\omega$ -3 fatty acids that comprise cell membranes by way of a parenteral injection of fatty acid triglycerides in the form of an isotonic lipid emulsion.	Yvon A. Carpentier Isabelle E. Dupont	01117991 USA
Nutritional formula for ICU patients	Provides a formula for administration to intensive care patients who have weakened states of absorption capacity. The formula is given enterally and consists of a source of protein, carbohydrate, and $\omega$ -3/ $\omega$ -6 fatty acids.	John Alexander et al.	96202637 USA
Formulation for smokers	Formula for combating the negative effects of smoking, such as aging of the skin; consists of a combination of $\omega$ -3 and $\omega$ -6 fatty acids.	David F. Horrobin	94301853 USA
Pharmaceutical composition for morbid affections	A composition containing esters of $\omega$ -3 PUFAs that have proven to be clinically useful in the treatment of psoriasis and phlebitis.	Tiberio Bruzzese et al.	93110903 USA
Formulation for protective effect on the liver	A combination of $\omega$ -3 fatty acids in their esterified form accompanied by medium-length triglycerides. The triglycerides are preferentially oxidized and thus protect the fatty acids from rapid oxidation. This combination helps to protect the liver and suppresses chronic inflammatory disorders.	Dr. Jorg Nehne Michael Boll	88116623 USA
Emulsion containing $\omega$ -3 fatty acids for treating inflammatory diseases	An emulsion that contains multiple $\omega$ -3 fatty acids or their esters along with traditional additives for the treatment of ulcerative colitis.	Jeffrey Askanazi et al.	00637957/EP B1
Drinkable $\omega$ -3 preparation	A liquid nutritional drink consisting of $\omega$ -3 PUFAs in a water-based solution that does not turn rancid with time	Johan Myhre AS Coromar	00147377 WO
Composition for maintenance of gut integrity	A method for restoring gut integrity by administering a combination of $\omega$ -3 and $\omega$ -6 PUFAs.	Susan Marie Kaup	00035443 WO

risk of prostate cancer in humans [56]. Omega-3 PUFAs have also been shown to significantly reduce the incidence of tumor induction by dimethylbenz(a)anthracene in rats [57]. Addition of  $\omega$ -3 PUFAs to the cultures of lung carcinoma A427 [58], Hep2 human larynx tumor cells [59], pancreatic Mia-Pa-Ca-2 cells [60], and embryonal carcinoma Tera-2 cells [61] induces apoptosis in these cell lines. Omega-3 PUFAs also inhibit the growth of cervical cells immortalized by the highly oncogenic human papillomavirus 16 (HPV16), foreskin keratinocytes immortalized by HPV16, and keratinocytes grown from papillomas with an HPV etiology [62]. Furthermore, conjugated DHA with a triene structure has been shown to induce apoptosis in DLD-1 cells (colorectal adenocarcinoma) without any effect on normal human fibroblast cell lines [63]. While there are many examples of  $\omega$ -3 PUFAs-induced apoptosis, at present, the cellular and molecular mechanisms are unclear and a better understanding of the basic actions of  $\omega$ -3 PUFAs will be needed before these polyunsaturated fatty acids can be fully employed in the clinic as anticancer agents [64, 65].

Many anticancer drugs exert their influence by inducing apoptosis. Apoptosis, or programmed cell death, is the physiological method by which unwanted or unneeded cells are eliminated during development or other biological processes [66]. It is also an important process in degenerative diseases, autoimmune disorders, and neoplasia development [67]. As a genetically regulated mechanism, apoptosis can occur through many pathways, but it is defined by several typical cellular and molecular events, including cell shrinkage, endoplasmic reticulum dilation, membrane blebbing, and extensive nuclear fragmentation [66]. Caspases, a family of cysteine proteases, play a critical role in apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis [68-71].

#### OMEGA-3 FATTY ACIDS AND CYTOSOL-LINKED APOPTOSIS

Omega-3 PUFAs exert their anticancer effects by slowing down the growth of cancer cells via inhibition of cell cycle

Table 5. Functional Foods Containing  $\omega$ -3 PUFA<sup>3</sup>

Product Type	Product	Manufacturer	$\omega$ -3 content/serving
Cereal	Healthy Scoop	Food by Design www.foodbydesign.com	2800 mg
Cereal	Cranberry Cereal Almonds Cereal Apple Cinnamon	Zoe Foods www.zoefoods.com	2400 mg
Cereal Bar	Healthy Break	Food by Design	2800 mg
Cereal Bar	Zoe Flax and Soy Bar -chocolate -peanut butter -apple crisp -lemon	Zoe Foods	1500 mg per bar for chocolate and peanut butter bars; 2200 mg per bar for apple crisp and lemon bars.
Cookies	Flax Macs	Food by Design	2400 mg
Eggs	Born 3 eggs	Born 3 Marketing Corp www.born3.com	400 mg
Mix	Flax Jacks (pancake and waffle mix)	Food by Design	5000 mg
Oil	Golden Omega-Omega oil	Naturalways www.naturalways.com/omega-omega	5750 mg

<sup>3</sup>Food websites found using www.flaxcouncil.ca/foodlist.

progression. However, in the continued presence of  $\omega$ -3 PUFAs these arrested cells start dying thorough apoptosis. Previously, we demonstrated that DHA prolongs the S phase in cultured spleen lymphocytes [72]. Subsequently, other investigators demonstrated that  $\omega$ -3 PUFAs arrest malignant cells in the S phase [73] and prevent G1/S progression in HT-29 human colonic cells [74], vascular smooth muscle cells [75], and urothelial cells [76]. These observations indicate that  $\omega$ -3 PUFAs, particularly DHA, can exert their anticancer effects by arresting cell cycle progression. To date, however, little is known about the molecular and cellular events that lead to  $\omega$ -3 PUFA-mediated cell cycle arrest and subsequent apoptosis. Progression through each phase of the cell cycle is tightly regulated and involves the expression and rapid degradation of the cyclin-dependent kinase (cdk) complex. In general, the levels of cdk are relatively constant throughout the cell cycle, while the cyclin levels vary substantially [77]. Cyclin A appears in the S phase with the

onset of DNA synthesis [78]. Cyclin A associates initially with cyclin-dependent kinase-2 (cdk2) and later with cdk1 (also known as cell division control protein 2 or cdc2 and is involved in G2/M progression) [78]. This association, and hence the activities of cdk2 and cdc2, are essential for progression through the S phase to the G2 phase. Many of the effects of cyclin-cdks are mediated through phosphorylation of retinoblastoma protein (pRb) Fig. (3). pRb controls the progression of the cell cycle by regulating the activities of transcription factors, most importantly, E2F2 and E2F3. In a hypophosphorylated state, pRb physically associates with these transcriptional factors and blocks their ability to activate the gene expression of products necessary for cell cycle progression. Once phosphorylated, pRb loses much, if not all, of its growth inhibitory power and permits the advance into late G1, and hence, into the remainder of the cell cycle [79].

Table 6. Fatty Acid Composition of Selected  $\omega$ -3 Supplements

	Monounsaturated	Omega 3 (mg/g oil)	Omega-6	w-3/w-6 ratios
Sundown Flax Oil	190 (150)	555 (530)	140 (129)	3.96
Sundown Cod- Liver Oil	165 (???)	266 (160)	21.3 (???)	12.48
Sundown Fish Oil	388 (???)	243 (300)	20 (???)	12.15
Puritan's Pride	73 (???)	527(500)	28(???)	10.51
Nature Made	123 (???)	286 (360)	91 (45)	3.16
Member's Mark	138 (???)	323 (300)	30 (???)	10.7
Sigma Chem. Co.	160 (120-260)	313 (180-30)	43 (<60)	7.28

Number in parenthesis indicates the manufacturer's reported values on the label.

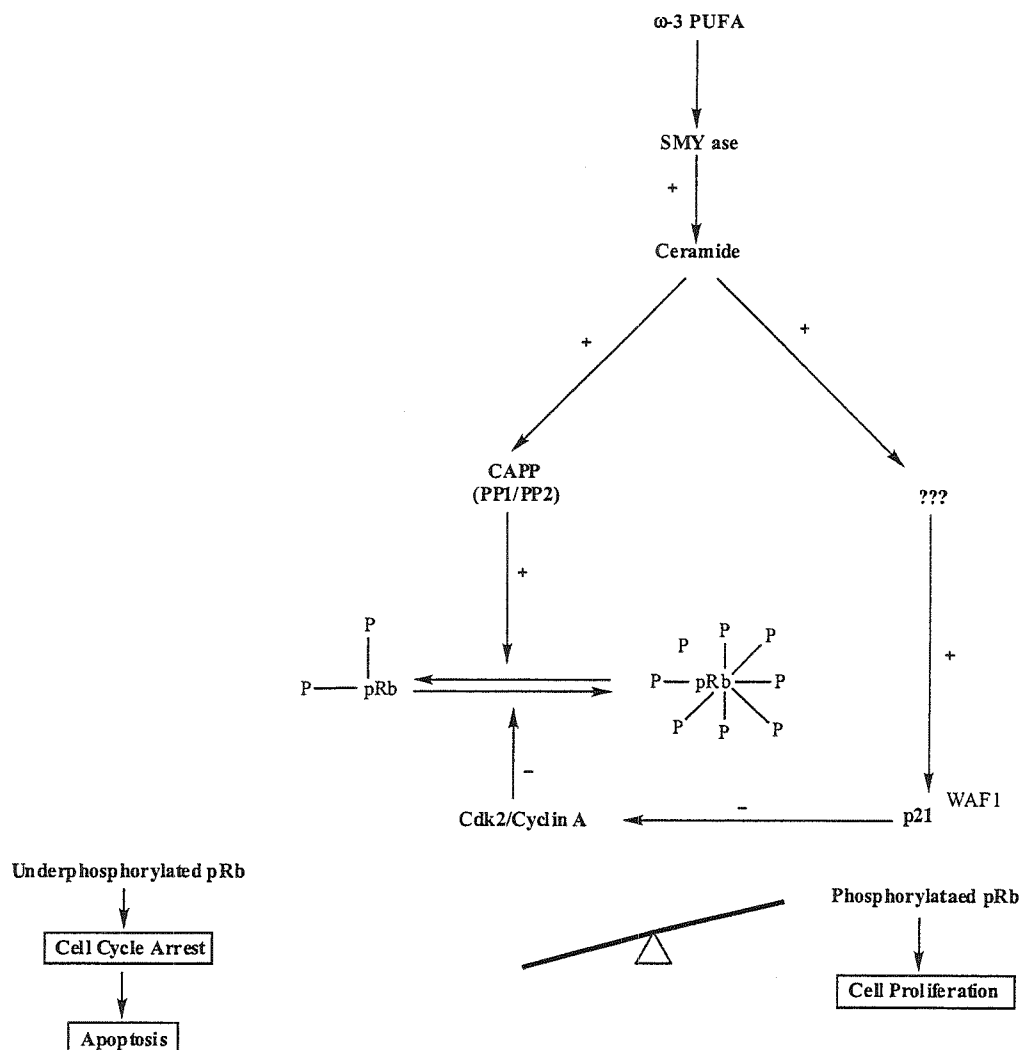


Fig. (3). Proposed action of  $\omega$ -3 PUFAs on cell cycle arrest in cancer cells

Incorporation of  $\omega$ -3 PUFAs into the cell membrane causes activation of sphingomyelinase (SMYase) and generation of ceramide. Ceramide mediates its effects via activation of ceramide-activated protein phosphatases (CAPP). Activation of CAPP (PP1 and/or PP2A) results in dephosphorylation of pRb phosphorylation. Ceramide also causes increased expression of p21<sup>WAF1</sup>, and subsequently, inhibition of cyclin A/cdk2 activities. The overall effect of DHA results in hypophosphorylation of pRb protein and cell cycle arrest.

For the past 15 years our laboratory has investigated the relationship of DHA's alteration of membrane structure to its effects on cell signaling and apoptosis. Our initial studies used model lipid bilayers to explore the interaction of DHA with membrane phospholipids [80-85]. These experiments were then extended to Jurkat leukemia cells to elucidate the mechanism of the anticancer effects of DHA [86-88]. We demonstrated that low doses of DHA induce S-phase cell cycle arrest in Jurkat cells through hypophosphorylation of pRb by inhibiting cdk2 kinase activity and stimulating protein phosphatase activities [88]. Our earlier studies using a model lipid bilayer suggest that DHA incorporation into membranes containing sphingomyelin and cholesterol affects

the formation of a type of lipid microdomain known as "lipid rafts" [39]. Lipid rafts may also contain sphingomyelinase (SMYase), an enzyme that generates ceramide, a potent second messenger involved in cell cycle arrest and apoptosis [89, 90]. Ceramide levels also change during progression of the cell cycle [91]. We therefore examined the levels of ceramide in DHA-induced growth-arrested cells. As predicted, our data demonstrate that DHA causes increased ceramide formation [92], probably resulting from DHA-induced activation of SMYase in the plasma membranes. Ceramide is a known potent activator of a protein phosphatase specific for cdk2 [92] and also functions as a modulator of pRb phosphorylation [91]. Furthermore, our research has demonstrated that DHA treatment of Jurkat cells leads to the activation of protein phosphatase 1 (PP1) and 2A (PP2A) [86, 87]. Therefore, it appears that DHA-induced ceramide formation leads to activation of protein phosphatases and then, subsequent to these events, dephosphorylation of pRb. Our studies also indicate that DHA induces elevated levels of p21<sup>WAF1</sup>. Ceramide has been shown to enhance expression of p21 [92], a cellular inhibitor of cdk2 kinase. Through the p21 mechanism, it is possible that elevated levels of ceramide lead to inhibition of

cdk2 kinase. It therefore appears that DHA-induced ceramide may regulate phosphorylation of pRb by directly activating protein phosphatases and perhaps by inhibiting cyclin A/cdk-2 activities via increased expression of p21<sup>WAF1</sup>. Although we have not yet studied the molecular mechanism by which ceramide leads to an increased expression of p21<sup>WAF1</sup>, it is clear that PP1 is not an involved upstream of p21<sup>WAF1</sup> expression. A role for ceramide in the induction of p21 via activation of nuclear factor kappa-B (NFκB) and/or p53 has been established by various studies [93, 94]. A possible mechanism for DHA-induced cell cycle arrest is outlined in Fig. (3).

Furthermore, we observed that growth-arrested cells undergo apoptosis upon repeated treatment with low doses of DHA. This apoptosis process appears to be mediated via caspase-3 activation [88]. Previously we suggested that activation of caspase-3, and hence, induction of apoptosis by

DHA, is also mediated through activation of protein phosphatases [86]. Our studies are consistent with several others that have shown that apoptosis can be mediated by activation of protein phosphatases. For example, Wolf and Eastman [95] demonstrated that activation of PP1 plays an important role in Fas-induced apoptosis by stimulating mitochondrial release of cytochrome C and caspase activation in HL-60 and Jurkat cells. Similarly, activation of a PP2A-like phosphatase has been demonstrated to play a key role in inducing apoptosis in a neuronal cell line [96]. Other studies have shown that CAPP, a member of the PP2A family, is involved in receptor-mediated induction of apoptosis in various cell lines [97]. These studies suggest that protein phosphatase activation may be a common feature of cells undergoing apoptosis (Fig. (4)). However, at present it is not clear how DHA activates protein phosphatases and how activation of protein phosphatases is linked to cell cycle arrest and induction of apoptosis. We did not test the role of

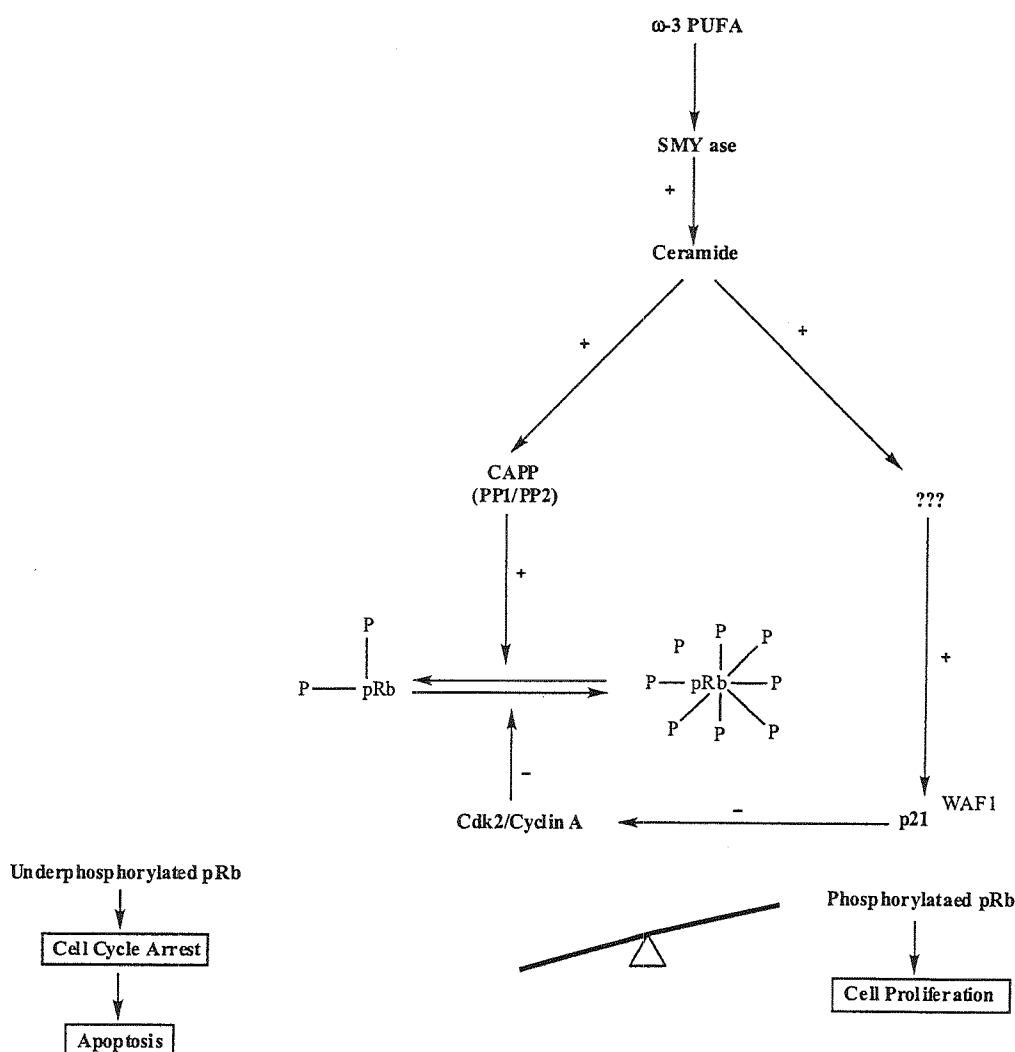


Fig. (4). Possible  $\omega$ -3 PUFA-induced involvement of protein phosphatase in apoptosis. Activation of protein phosphatases by  $\omega$ -3 PUFA-induced ceramide formation can affect cancer cell growth through multiple pathways. Via dephosphorylation of retinoblastoma protein (pRb) protein phosphatases cause cell cycle arrest, which then leads to the induction of apoptosis. Protein phosphatases can also mediate release of cytochrome c and activation of caspases via dephosphorylation of Bcl2/Bid proteins. However, it is also possible that protein phosphatases play a direct role in the activation of caspase 3. Activation of the executionary caspase 3 then leads to the induction of apoptosis.

EPA in cell cycle arrest during this investigation. EPA can be converted to DHA and therefore, it can have effects similar to DHA on cell cycle arrest. Indeed several investigators have demonstrated that EPA also blocks cell cycle progression and induces apoptosis in Ramos cells [98], squamous cell carcinoma [99], vascular smooth muscle cells [75], HT29 colonic cells [100], and pancreatic PaCa-2 cancer cells [101]. In most cases the results are consistent with our finding of growth arrest in the S phase of cell cycle progression [75, 100, 101] through inhibition of cdk2 activities [75].

While our studies probed one possible pathway for DHA's effect on cell growth and viability, many other, often overlapping possible modes of action undoubtedly exist.

### OMEGA-3 FATTY ACIDS AND MITOCHONDRIA-LINKED APOPTOSIS

The involvement of mitochondria in apoptosis has been demonstrated by several investigators in recent years [102-107] and this pathway has also been strongly implicated in  $\omega$ -3 PUFA-induced cell death. Omega-3 PUFAs have been reported to alter mitochondrial membrane properties and functions in rat colonocytes [108], the human colon tumor cell line HT29 [109], Walker 256 rat carcinosarcoma [49], T24 [49], and Hep2 [59] cancer cells.

Evidence suggests that DHA, but not EPA, preferentially accumulates in cardiolipin [109]. Cardiolipin (CL) is a diphospholipid (diphosphatidylglycerol) required for mitochondrial structural integrity and for the proper function of the electron transport chain [110]. CL is absent from all other cell membranes other than mitochondria, where it is present in the inner membrane and at intermembrane contact sites [110]. In tissues with high respiration rates, such as heart, CL can account for 25% of the phospholipids in the inner-mitochondrial membrane [111], where it is usually bound to the enzyme complexes of electron transport and ATP synthesis (i.e. cytochrome *c* oxidase [112-114], NADH reductase [115, 116], cytochrome *b<sub>7</sub>c<sub>1</sub>* complex [116, 117], and ATP synthase) [117, 118]. This suggests that mitochondrial function is very much dependent on the proper amount of CL. The CL acyl composition is sensitive to diet, and in humans it is usually rich in the essential dietary fatty acid linoleic acid (LA, 18:2 n-6) [119]. However, any change in dietary fatty acids is reflected in a change in acyl composition of CL. In mammals, CL has been modified to contain 85-90 mol% LA [120, 121], 50 mol% DHA [122], or 50 mol% oleic acid (OA) [122]. It is believed that  $\omega$ -3 PUFAs in CL are susceptible to reactive oxygen species (ROS), which are generated through oxidative phosphorylation. CL is peroxidized by ROS and this process results in a decrease in CL levels in the mitochondrial membrane [123]. It has been suggested that low levels of CL either by peroxidation or its decreased synthesis [124, 125] compromises the integrity of CL-dependent proteins involved in energy metabolism, causing a drop in mitochondrial membrane potential, which in turn initiates apoptosis [126].

Consistent with these suggestions, cancer cells treated with  $\omega$ -3 fatty acids clearly exhibit alterations in mitochondrial membrane potential and undergo apoptosis

[49, 59, 108, 109]. Recently, a number of studies have reported a mechanism of mitochondrial-mediated apoptosis. It has been suggested that the peroxidation and/or loss of CL induces cytochrome C release from mitochondria. Studies have shown a highly significant temporal correlation of CL depletion with cytochrome C release to the cytosol [110]. The integrity of mitochondria and release of cytochrome C are regulated by Bcl-2 family members residing in the outer mitochondrial membrane [127]. Bcl-2 family members encode proteins that can be either antiapoptotic (e.g., Bcl-2, Bcl-X<sub>L</sub>) or pro-apoptotic (e.g., Bax, Bcl-X<sub>S</sub>, Bak, Bad, Bid) and therefore integrate signals from growth and death stimuli. An excess of Bcl-2 antiapoptotic proteins over Bcl-2 proapoptotic proteins protects the integrity of mitochondria and prevents cytochrome C release, whereas an excess of proapoptotic Bcl-2 proteins over antiapoptotic Bcl-2 proteins allows leakage of cytochrome C. It has been demonstrated recently that one chain of CL is inserted into a hydrophobic channel in cytochrome C, whereas another acyl chain extends into the bilayer [128]. CL is released from mitochondria for degradation in peroxisomes [129]. The proapoptotic protein Bid plays a role in the transfer of CL [130]. It has been shown that CL transfer occurs at the same concentrations of Bid that lead to mitochondrial release of cytochrome C [130]. The activities of the Bcl-2 family are regulated by different mechanisms, such as homo- and heterodimerization with other family members and also by proteolysis and phosphorylation [131]. There are recent reports that antiapoptotic Bcl-2 is inactivated by phosphatases, particularly by PP2A [132]. We have demonstrated that DHA treatment of Jurkat leukemia cells results in ceramide formation [88], which is also known to activate a phosphatase with characterised PP2A-type properties [97]. Similarly, dephosphorylation of Bad (proapoptotic) results in its activation and binding to Bcl-2, initiating cytochrome C release. The release of cytochrome C then interacts with Apaf-1 and dATP, leading to caspase 9 activation and hence downstream execution of the caspase cascade [133, 134]. The effector caspases are active proteases that then lead to morphological changes characteristic of apoptotic cell death, such as membrane blebbing and formation of apoptotic vesicles, cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation. The omega-3-induced steps in mitochondria-linked apoptosis are outlined in Fig. (4).

### SUMMARY

Epidemiological and dietary studies strongly indicate that  $\omega$ -3 PUFAs provide tremendous health benefits for a number of diseases including cancer and heart disease.  $\omega$ -3 PUFAs are readily obtained from naturally occurring foods, manufactured functional foods, or from  $\omega$ -3 PUFA supplements. One has to be careful, however, as our study suggests that different brands of supplements contain widely different amounts of  $\omega$ -3 PUFAs and other mono and polyunsaturated fatty acids and cholesterol.  $\omega$ -3 PUFAs have been shown to alter biologically essential processes including cell signaling and apoptosis. Here we reviewed how  $\omega$ -3 PUFAs might regulate cellular signaling pathways and induce apoptosis through cytosolic and mitochondrial mediated signaling pathways. But even this is

controversial, as  $\omega$ -3 PUFAs have been shown to prevent apoptosis in heart, neuronal, and retinal tissues. In these organs,  $\omega$ -3 PUFAs appear to preserve function and exhibit anti-apoptotic properties through similar cellular signaling pathways that induce apoptosis in other organs. Comparing details of the effects of  $\omega$ -3 PUFAs on cell signaling in different tissues therefore offers a unique approach in developing  $\omega$ -3 PUFA-containing drugs. These drugs may selectively destroy cancer cells while preserving the vital physiological functions of other healthy tissues.

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## Role of omega-3 fatty acids in the prevention of cancer-induced muscle proteolysis

### CHED 242

Heidi R. Yount and Rafat A. Siddiqui. Cell Biochemistry Lab, Methodist Research Institute, 1813 N. Capitol Ave., Indianapolis, IN 46201

Cancer growth causes many detrimental effects on the host system. One of the contributing factors in cancer mortality is loss of lean body mass due to muscle wasting (cachexia). Muscle hypercatabolism occurs via three proteolytic pathways: Calpain (calcium dependent neutral protease) pathway, ATP-Ubiquitin proteasome pathway, and the lysosomal pathway. As tumors grow, soluble proteolysis-inducing factors which induce proteolysis by activating calpain enzymes are released. This experiment tested Omega-3 fatty acids, primarily docosahexaenoic acid (DHA), to determine the effect on tumor-induced muscle wasting through inhibition of the calpain pathway. Calpain-mediated proteolysis pathway involvement was investigated using an in vitro system, including skeletal muscle and cardiac muscle cells. Muscle proteolysis in these cells was induced by the growth media of different cancer cells, including: leukemia (Jurkat), breast cancer (MDA-231-MB), cervical cancer (HeLa), and colon cancer (T84). The effect of DHA on reducing muscle proteolysis was tested by treating the muscle cells with DHA prior to treatment with the cancer cell growth media. DHA inhibited the proteolysis induced by breast cancer cells. Activation of the calpain pathway was studied in skeletal muscle cells. The growth media from the MDA, HeLa, and T84 cells all induced calpain activation in the untreated cells, while in the cells treated with DHA, no activation was noted. Following the in vitro studies, the effect of dietary supplementation of w-3 fatty acids on breast cancer cell (MDA-231-MB) growth and tumor-induced muscle proteolysis was investigated. Breast cancer cells were implanted in nude mice which were either fed corn oil rich diets (Group 1, 1:18 w-3 FA:w-6 FA), fish oil rich diet (Group 2, 2.6:1 w-3 FA:w-6 FA), or a balanced mixture of corn oil and fish oil (Group 3, 1:1 w-3 FA:w-6 FA) for three weeks prior to and three weeks post tumor implantation. Tumor growth and calpain activity of the cardiac and skeletal muscles was reduced in both Group 2 and Group 3 compared to Group 1. Overall, this study suggests that cancer cells release soluble proteolysis-inducing factor(s) that induce proteolysis by activating calpain enzymes. w-3 FA inhibit the calpain-associated proteolysis pathway and dietary supplementation of w-3 offers a preventive effect against muscle wasting associated with cancer.

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**Press Releases: Wu M, Harvey KA, Ruzmetov N, Welch Z, Sech L, Jackson K, Stillwell W, Zaloga GP, Siddiqui, RA. (2005). Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway. *Intl. J. Cancer* 117, 340-348**

### **Dietary fish oil curbs breast cancer progression in animal study**

*Last Updated: 2005-11-24 12:44:08 -0400 (Reuters Health)*

NEW YORK (Reuters Health) - The growth of breast cancer cells in culture and in mice is inhibited by omega-3 fatty acids, scientists report in a fast track article in the November 10th International Journal of Cancer.

According to Dr. Rafat A. Siddiqui from the Methodist Research Institute, Clarian Health Partners in Indianapolis, "Omega-3 fatty acids activate an enzyme called sphingomyelinase, which generates the release of ceramide, a compound that ultimately causes cancer cell death."

Most American and British diets are high in omega-6 fatty acids (common in beef products) and low in omega-3 fatty acids (common in fish oils), noted the researcher. In a number of human studies, diets rich in omega-3 fatty acids were associated with a lower risk of breast and colon cancers. However, the mechanisms by which these dietary fats affect development and growth of cancers has been unclear.

Dr. Siddiqui and colleagues set out to determine if dietary lipids could modulate growth of breast cancer cells in animals and to determine the cellular mechanisms by which dietary lipids alter growth of breast cancer cells.

In the animal study, mice were fed diets rich in omega-3 (fish oil) or omega-6 (corn oil) fatty acids. Three weeks after implantation of breast cancer cells, tumor volume and weight were significantly lower in the omega-3 group compared with the omega-6 group.

The omega-3-rich diet also led to a 40% increase in sphingomyelinase and increased expression of p21, which is associated with growth arrest.

Similar results were seen in cultured breast cancer cells.

"Our study shows that dietary intake of different fatty acids affects the growth of breast cancer cells," Dr. Siddiqui said.

"Dietary fatty acids," he explained, "are incorporated into cell membranes, and the type of fatty acids dictates the localization of key signaling molecules within the cell. These signaling molecules then regulate cell growth."

"Our studies indicate that sphingomyelinase, an enzyme usually present in lipid rafts located within the cell membrane, changes its localization in the cell in the presence of omega-3 fatty acids, possibly due to changes in the structure of the lipid rafts," the researcher said.

This study, Dr. Siddiqui concluded, "suggests that incorporating moderate amounts of omega-3 fatty acids into the diet may decrease breast cancer progression. Importantly, omega-3 fatty acids are already known to have beneficial cardiovascular effects in humans."

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## Fish oil shows promise in treating cancer

Anne Marie Tiernon/Health Reporter

Indianapolis, Nov. 29- "I was diagnosed six months ago and I had stage four primary breast cancer." Melody West-Marple is in the fight of her life, a pea size growth on her clavical diagnosed as breast cancer.

"It has changed my whole life."

After chemotherapy, radiation and hormone therapy are ahead.

Melody is changing her diet too. "I'm doing the the supplements, the vitamins, the diet, the exercise, I'm doing all this stuff to fight it."

That's exactly what Methodist researcher Rafat Siddiqui says she should do after his US Department of Army research funded study of Omega 3 fatty acids found in fish oil. "What we found was including a little bit of Omega 3 fatty acid in diet actually slows down the growth of cancer."

Pictures illustrate his findings. In rats transplanted with breast cancer tumors, when given corn oil the tumor is red, swollen and larger. The tumor is smaller in the rat given a combination corn and fish oil. And it's hard to detect in the rat given fish oil alone.

"By changing your diet," says Siddiqui, "you basically change the composition of your cells."

That change in breast cancer cells disturbs the growth signal and inhibits the metasis, or movement of cancer cells from one place to another.

A chart shows the decline, inhibited cancer cell growth from 25 to 80 percent.

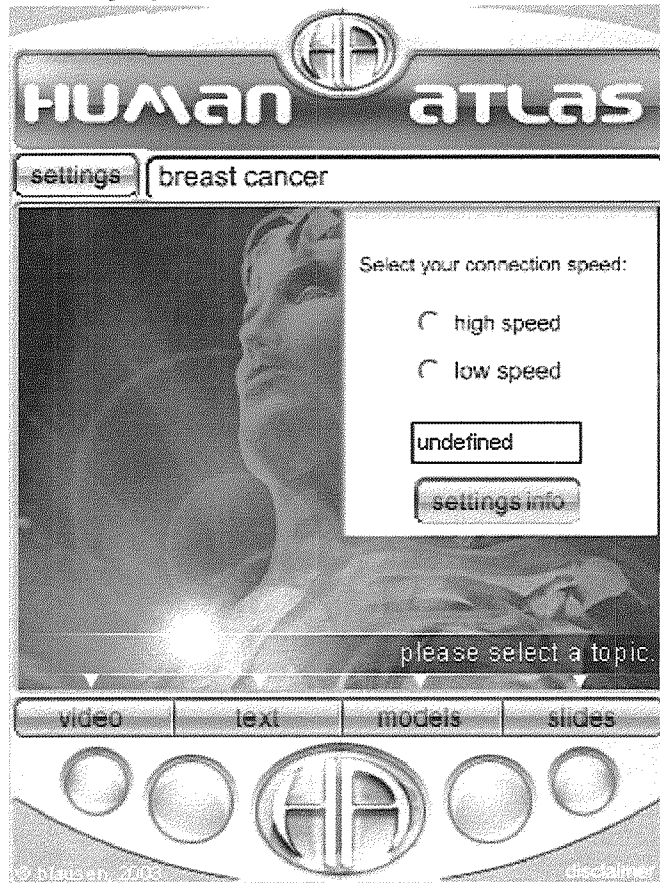
At your local Kroger you can get fish oil in tablet form. Fish like wild salmon and tuna, not

light but white, gives twice the punch. Eggs are now fortified. Cereals from regular to are all ways to get your one to two grams of Omega 3 a day.

Melody says "If that is what I have to do to stay around, then that is what I am going to do. I am too young to die."

### 3D Interactive Human Atlas

Now Playing: breast cancer



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To start the Interactive Human Atlas, click on the buttons labeled "Video", "Text", "Models" or "Slides."

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## FAST TRACK

# Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway

Min Wu<sup>1</sup>, Kevin A. Harvey<sup>1</sup>, Nargiz Ruzmetov<sup>1</sup>, Zachary R. Welch<sup>1</sup>, Laura Sech<sup>1</sup>, Kim Jackson<sup>2</sup>, William Stillwell<sup>2</sup>, Gary P. Zaloga<sup>1,3</sup> and Rafat A. Siddiqui<sup>1,2,3\*</sup>

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The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. In *in vivo* experiments, mice were fed diets that were rich in either omega-3 (fish oil) or omega-6 (corn oil) fatty acids. Three weeks after implantation of MDA-MB-231 breast cancer cells, the tumor volume and weight were significantly lower ( $p < 0.05$ ) for mice fed the omega-3 diets compared to those fed the omega-6 diets. Dietary fish oil also caused a 40% ( $p < 0.05$ ) increase in neutral sphingomyelinase (N-SMYase) activity in the tumors. The tumor tissues from fish oil-fed animals expressed elevated p21 (waf1/cip1) mRNA, whereas tumor tissues from corn oil-fed animals exhibited undetectable levels of p21 expression. In *in vitro* experiments, at concentrations as low as 25  $\mu$ M, DHA and EPA inhibited the growth of cultured MDA-MB-231 cells in a dose-dependent manner by 20–25% ( $p < 0.05$ ). N-SMYase activity was also increased by 30–40% ( $p < 0.05$ ) in the DHA- or EPA-treated cells in which an increase in ceramide formation was observed. DHA and EPA were both observed to enhance membrane bleb formation and also to induce the expression of p21. Omega-3 fatty acids-induced bleb formation and p21 expression were inhibited by the N-SMYase inhibitor GW4869, which also inhibited apoptosis by approximately 40% ( $p < 0.05$ ). The results suggest that inhibition of breast cancer growth in nude mice by dietary fish oil and inhibition of breast cancer cell growth in culture by treatment with DHA and EPA is mediated by activation of N-SMYase.

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**Key words:** breast cancer; docosahexaenoic acid; eicosapentaenoic acid; fish oil; sphingomyelinase; ceramide

The oils of certain cold-water fish have a well-documented role in inhibiting or preventing cancer. Epidemiologic evidence strongly links fish oil with low incidences of several cancers.<sup>1–4</sup> The anticancer properties of fish oils have been attributed to the omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Proof of these fatty acids as anticancer agents has been substantiated by dietary studies on many types of animals, including humans and in cultured cells.<sup>5–14</sup> A number of studies have indicated that DHA's anticancer properties are not directly due to cytotoxicity but rather to the fatty acid's ability to induce apoptosis.<sup>15–18</sup> However, the molecular mechanism for the anticancer actions of omega-3 fatty acids remains unknown. Understanding the mechanistic effects of omega-3 lipids may aid in the development of new cancer therapies.

Numerous studies, including our own,<sup>5,19–24</sup> have linked fish oil to induction of apoptosis. We found that DHA activates sphingomyelinase (SMYase) activity in the plasma membrane of Jurkat leukemic cells, increasing ceramide levels.<sup>23</sup> SMYase is an enzyme that catalyzes the hydrolysis of sphingomyelin (SM) to ceramide. A variety of studies have shown that ceramide is ubiquitously produced during cellular stress and is associated with apoptosis.<sup>25,26</sup> To date, at least 7 classes of mammalian SMYases have been described, differing in subcellular location, pH optimums, cation dependence and roles in cell regulation.<sup>27–29</sup> However, only 2 forms of SMYases, distinguishable by their pH optima, are capable of initiating signal transduction.<sup>30</sup> The acid SMYase (pH opti-

mum 4.5–5.0) is a cellular glycoprotein located in the acidic lysosomal compartment where it contributes to lysosomal SM turnover.<sup>31</sup> The neutral SMYase (N-SMYase; pH optimum 7.4) is a plasma membrane-bound enzyme<sup>32,33</sup> that has been implicated in mediating apoptosis in cells exposed to stressing agents. Substantial amounts of N-SMYase are proposed to reside in "lipid rafts."<sup>34</sup> Therefore, factors influencing the lipid composition of membranes can influence the activity and distribution of N-SMYase in "lipid rafts." We have demonstrated that DHA may alter lipid raft formation<sup>35</sup> and induce SMYase activity, leading to cell-cycle arrest in leukemic cells.<sup>23</sup> Furthermore, treating cells with synthetic short-chain ceramide has been shown to induce cell-cycle arrest and apoptosis.<sup>36</sup> Ceramide levels also change during progression through the cell cycle<sup>37</sup> and have been shown to enhance expression of p21 (waf1/cip1),<sup>38</sup> a cellular inhibitor of cdk2 kinase that is involved in cell-cycle arrest via hypophosphorylation of retinoblastoma protein (pRb).<sup>39</sup> We have previously shown that DHA-induced ceramide may regulate phosphorylation of pRb by inhibition of cyclin A/cdk-2 activities via increased expression of p21.<sup>23</sup> In our study described below, we investigated growth inhibition of breast cell xenografts by dietary fish oil in nude mice and the cellular effects of DHA and EPA in cultured breast cancer cells. Our data demonstrate that the long-chain omega-3 fatty acids inhibit breast cancer growth by activating N-SMYase, thereby generating ceramide.

## Material and methods

### Material

Human breast cancer MDA-MB-231 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). Nude mice (nu/nu) were purchased from Charles River Laboratories (Wilmington, MA). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin and glutamine were from Invitrogen (Grand Island, NY). Fetal bovine serum was from BioWhittaker (Walkersville, MD). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), oleic acid (OA), linoleic acid (LOA) and fatty acid standards for gas chromatography (GC) were from Nu-Check Prep (Elysian, MN). Annexin V staining, cell death detection ELISA, WST-1 assay and the lactate dehydrogenase kits were

**Abbreviations:** DAPI, 4', 6-Diamidino-2-phenylindole; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LOA, linoleic acid; N-SMYase, neutral-sphingomyelinase; OA, oleic acid; p21, waf1/cip1; PS, phosphatidylserine.

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TABLE I – EXPERIMENTAL DIETS

	n-6 diet	n-3 diet
Carbohydrates (% calories)	58.8	58.8
Starch (g/100 g)	25.0	25.0
Maltodextrins (g/100 g)	5.0	5.0
Sucrose (g/100 g)	30.0	30.0
Cellulose (g/100 g)	5.0	5.0
Protein (% calories)	19.6	19.6
Casein (g/100 g)	20.2	20.2
DL-methionine (g/100 g)	0.3	0.3
Lipid (% calories)	21.7	21.7
Corn oil (g/100 g)	10.0	1.0 <sup>1</sup>
Fish oil (g/100 g)	0	9.0 <sup>1</sup>
n-6/n-3 ratio	72.5	0.11 <sup>1</sup>
Mineral mix (g/100 g)	3.45	3.45
Vitamin mix (g/100 g)	1.0	1.0
Vitamin E (g/100 g)	0.03	0.03
DHT (g/100 g)	0.02	0.02

<sup>1</sup>Differs between diets.

purchased from Roche Biochemicals (Indianapolis, IN). N-SMYase inhibitor, GW4869, was from Calbiochem (San Diego, CA). Hanks Balanced Salt Solution (HBSS), the fish oil (Manhaden) and all other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO).

#### Animal studies

Nude mice (nu/nu; Charles River Laboratories) were fed *ad libitum* corn oil (omega-6/omega-3 ratio of 72:1), balanced corn oil/fish oil (omega-6/omega-3 ratio of 1:1), or fish oil (omega-6/omega-3 ratio of 0.11:1) diets (Research Diets, New Brunswick, NJ) for 3 weeks prior to tumor implantation. Diets contained similar quantities of protein (59% of calories), carbohydrates (20% of calories), lipids (21% of calories), vitamins, and minerals as described in Table I. They only differed in the types of lipids (*i.e.*, corn and fish oil), and their fatty acids composition is described in Table II. Tumor xenografts were implanted by injecting subcutaneously 200  $\mu$ l of MDA-MB-231 cells ( $1 \times 10^6$  cells) on the backs of animals, and the animals were returned to their corresponding diets for another 3 weeks. Tumor growth was monitored by measuring length and circumference of tumors by a flexible wire tape as described,<sup>40</sup> and tumor weight was determined at termination of the study after excising the tumor free of connective tissue. The tumor tissues were freeze-clamped in liquid nitrogen for later analysis.

#### Cell cultures

MDA-MB-231 breast cancer cells were grown in DMEM media containing 10% fetal bovine serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin at a density of  $1 \times 10^6$  cells/ml for routine culture. For experimental purposes, cells were cultured at the cell density indicated and treated with fatty acids under serum-free conditions. The fatty acids EPA, DHA, OA and LOA were stored in ethanol under liquid nitrogen and diluted in ethanol just prior to use. The final concentration of ethanol (< 0.1%) in the treated cultures did not induce any cytotoxic effects as measured by lactate dehydrogenase release and a WST-1 cell proliferation assay (results not shown).

#### Cell growth assay

The effect of the fatty acids on cell growth was determined using a WST-1 assay per the manufacturer's instructions (Roche Biosciences).

#### N-SMYase assay

Sphingomyelinase activity was measured by an Amplex Red sphingomyelinase assay kit (Molecular Probes, Eugene, OR). Briefly, the frozen tumor tissues were ground under liquid nitrogen and then homogenized in a reaction buffer containing

TABLE II – FATTY ACID COMPOSITION OF DIETARY OILS

Fatty acids	Corn oil (%)	Fish oil (%)
14:0		8.0
16:0	12.0	17.0
16:1		14.0
18:0	7.0	4.0
18:1n-9	22.0	26.0
18:2n-6	58.2	2.0
18:3n-3	0.8	3.0
18:4n-3		3.0
20:4n-6		1.0
20:5n-3		12.0
22:6n-3		10.0
Saturated fatty acids	19.0	29.0
Monounsaturated fatty acids	22.0	40.0
Total n-6 lipids	58.2	3.0
Total n-3 lipids	0.8	28.0
n-6/n-3 ratio	72	0.11

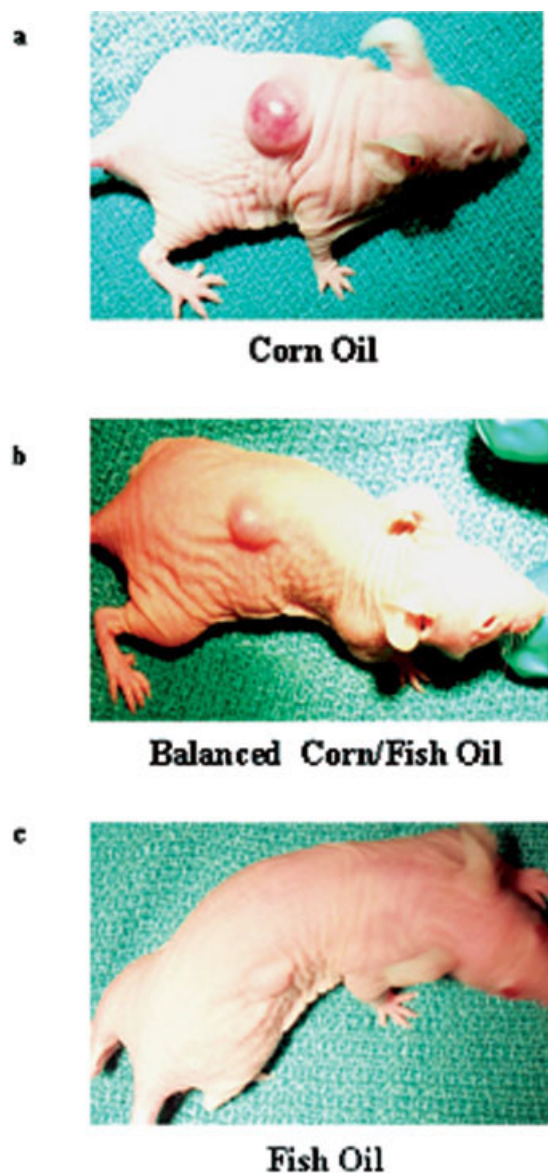
100 mM Tris-HCl (pH 7.5), 50 mM MgCl<sub>2</sub> and 0.1% Triton X-100. For measuring sphingomyelinase activities in MDA-MB-231 cells, DHA- or EPA-treated cells were lysed in 100  $\mu$ l of lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10% v/v glycerol, 1% Nonidet P-40, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mg/ml leupeptin, 0.15 units/ml aprotinin and 2.5 mM DIFP] for 10 min on ice. Protein concentrations in tumor homogenates, cell lysates and isolated membrane fractions (see below) were measured using a bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL), and samples were diluted in reaction buffer for the assay in the presence of exogenous sphingomyelin (0.25 mM). The released phosphorylcholine from sphingomyelinase activity was measured by the sequential activity of alkaline phosphatase and choline oxidase. The resultant release of H<sub>2</sub>O<sub>2</sub> was quantified by measuring fluorescence intensities (excitation at 540 nm and emission at 590 nm) after reaction with the Amplex Red reagent as described in the manufacturer's protocol. Amount of sample protein (approximately 10  $\mu$ g) resulted in a linear fluorescence intensity from approximately 1/5th to 1/10th of the positive control.

#### Analysis of p21 mRNA expression

RNA from tumor tissues or cultured cells was extracted by an RNA assay kit (Qiagen, Valencia, CA). The amount of RNA in an aqueous solution was determined by absorbance at 260 nm. Semi-quantitative RT-PCR was performed to determine the mRNA levels of p21 and GAPDH (loading control) using the Titan One Tube RT-PCR System (Roche Diagnostics). The primer sequences 5'-CGG-TCC-CGT-GGA-CAG-TGA-GCA-G3', 5'-GTC-AGG-CTG-GTC-TGC-CTC-CG3' were used for p21. The thermocycling parameters were composed of an initial cycle at 50°C for 30 min for reverse transcription of RNA into cDNA. The subsequent DNA amplification was performed with a thermocycling reaction consisting of 95°C for 180 sec followed by 30 cycles at 95°C for 60 sec, 55°C for 60 sec and 72°C for 60 sec.

#### Western blot analysis

After treatment with DHA or EPA, cell lysates were separated by SDS PAGE (10%), and then electro-blotted onto presoaked Immobilon-P membranes (Millipore, Bedford, MA) as described previously.<sup>23</sup> The membranes were blocked in 5% dry milk in TBS-T solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 2 hr at room temperature. The blot was incubated with monoclonal anti-p21 or monoclonal anti-GAPDH antibodies (Santa Cruz Biotech, Santa Cruz, CA; 1:1,000) at 4°C overnight and detected using secondary anti-rabbit peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK; 1:2,000 in TTBS). The bands were detected using a chemiluminescence detection kit (Pierce).



**FIGURE 1** – Fish oil inhibits breast cancer growth in mice. Nude mice were maintained on (a) corn oil (omega-6/omega-3 ratio 72:1), (b) balanced corn/fish oil (omega-6/omega-3 ratio 1:1) or (c) fish oil (omega-6/omega-3 ratio 0.11:1) diets for 3 weeks prior to subcutaneous implantation of MDA-MB-231 cells ( $1 \times 10^6$  cells). The animals were then further fed on the corresponding diets for another 3 weeks. Results are representations from 6 mice in each group.

#### Immunohistochemistry

Ceramide and p21 formation were determined using immunohistochemistry.<sup>41</sup> After incubation with serum-free media containing DHA or EPA, the cells were fixed with 3% paraformaldehyde and then blocked with 1% BSA in PBS. Ceramide was detected using a specific anticardiolipin antibody (Alexis, Carlsbad, CA; clone MID 15B4; 1:200 dilution in blocking buffer) and assayed using an Alexa 488-labelled anti-mouse antibody (Molecular Probes; 1:200 dilution in blocking buffer), whereas p21 was detected using anti-p21 (Santa Cruz Biotech; 1:200 dilution in blocking buffer) and assayed using an Alexa-546-labelled mouse antibody. Presence of the nuclei was detected by DAPI stain. Cells were examined under a fluorescence microscope and pictures were taken using a MagnaFire digital camera (Optronics, Goleta, CA) for analysis.

**TABLE III** – EFFECT OF CORN OIL AND FISH OIL DIETS ON BREAST CANCER GROWTH IN NUDE MICE

Diet	Tumor volume (mm <sup>3</sup> )	Surface area (mm <sup>2</sup> )	Tumor weight (mg)
Corn oil	307.7 $\pm$ 7.0	76.9 $\pm$ 3.2	180 $\pm$ 10
Balanced corn/fish oil fed	114.5 $\pm$ 17.3*	49.8 $\pm$ 7.4*	130 $\pm$ 20*
Fish oil	75.5 $\pm$ 6.9*	38.9 $\pm$ 3.3*	90 $\pm$ 10*

Results are the mean  $\pm$  SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the corn oil group are reported (\* $p < 0.05$ ).

#### Cell death ELISA

Quantitative analysis of DNA fragmentation was carried out using a histone-based Death ELISA system (Roche) per the manufacturer's protocol. MDA-MB-231 cells ( $1 \times 10^6$ /ml) in 6-well plates were incubated with fatty acids for 24 hr and then lysed. The nucleosomes containing fragmented DNA were captured by an immobilized antihistone antibody. The amounts of DNA fragments were then determined spectrophotometrically using a peroxidase-conjugated anti-DNA antibody.

#### Annexin V staining

Externalization of phosphatidylserine was evaluated using an annexin V staining kit (Roche Biochemicals) per the manufacturer's instructions. This kit can distinguish apoptotic and necrotic (dead) cells by propidium iodide (red) staining and FITC-conjugated annexin V (green) staining, respectively. The cells were visualized using a fluorescence microscope. The total and apoptotic cells were counted, and the percentage of cells exhibiting apoptosis was calculated.

#### Statistics

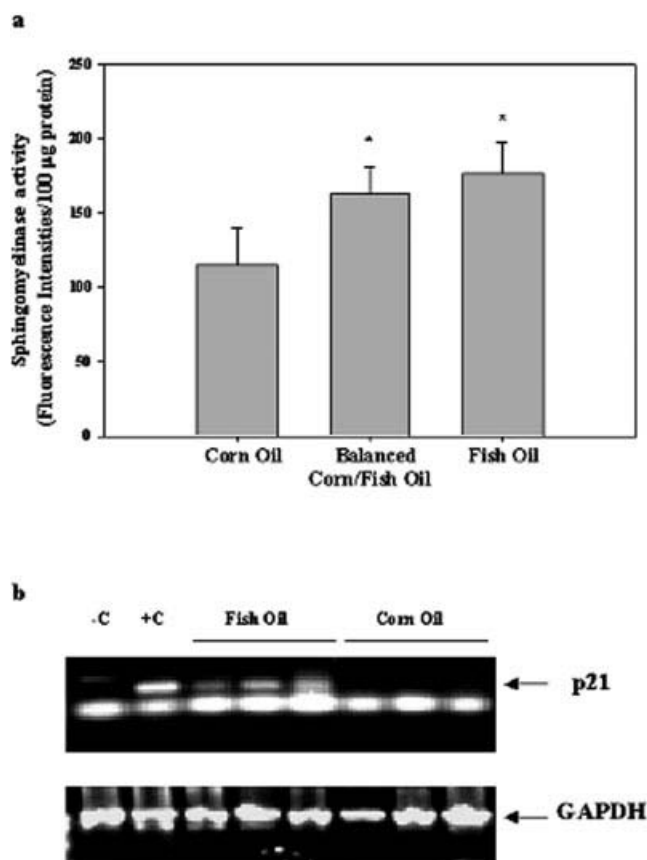
All experiments were performed at least 3 times each in triplicate and expressed as mean  $\pm$  SE. Comparisons were done using a Student's *t*-test and 1-way ANOVA. Significance was defined as  $p < 0.05$ .

#### Results

##### *Inhibition of breast cancer tumors by dietary fish oil (in vivo studies)*

The effect of dietary fish oil on breast cancer growth was investigated in nude mice. Results demonstrate that mice fed a corn oil diet rich in omega-6 fatty acids exhibited substantial tumor growth (Fig. 1a). In contrast, mice fed on the omega-6/omega-3-balanced diet exhibited reduced cancer growth (Fig. 1b). Tumor growth in mice fed the omega-3-rich fish-oil diet had by far the smallest tumors (Fig. 1c). Because the tumors were asymmetric, quantification was achieved by measuring tumor volume, surface area and mass. Data shown in Table III indicate that at 3 weeks after tumor implantation, tumor volume was approximately 60% lower ( $p < 0.05$ ), whereas tumor surface area and weight were approximately 30–40% lower ( $p < 0.05$ ) in mice maintained on the balanced diet (omega-3/omega-6, 1:1) compared to mice on the corn oil diet (omega-6 enriched). The largest reduction in tumor volume was noted for the fish-oil diet (omega-3 enriched), where tumor volume was approximately 75% lower ( $p < 0.05$ ), and tumor surface area and weight were approximately 50% lower ( $p < 0.05$ ) compared to animals maintained on corn oil diets.

Tumor tissue isolated from mice maintained on each of the 3 diets was then analyzed for N-SMYase activity. Data in Figure 2a show that N-SMYase activity was higher by approximately 40% ( $p < 0.05$ ) in the tissues from mice raised on the omega-3-containing diets (fish oil and balanced fish/corn oil) compared to those on the high omega-6 corn oil diet.

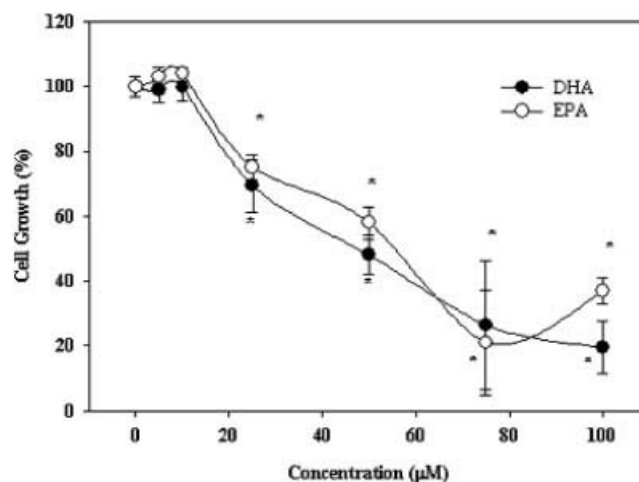


**FIGURE 2** – Fish oil enhances N-SMYase activity and p21 expression in tumor tissues. Mice implanted with breast cancer cells as described in Figure 1 were sacrificed and their tumor tissues were isolated. (a) N-SMYase activity was assayed as described in Material and Methods. Results are the mean  $\pm$  SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the corn oil group are reported (\* $p < 0.05$ ). (b) For p21 expression, RNA from tumor tissues were extracted and RT-PCR was performed to determine the mRNA levels of p21 and GAPDH (loading control) using the Titan One Tube RT-PCR System as described in Material and Methods. Negative control (-C) was muscle tissue from a normal mouse; positive control (+C) was RNA isolated from HCT101 p21<sup>+</sup> cells. Results are shown for 3 tumor tissues for each group.

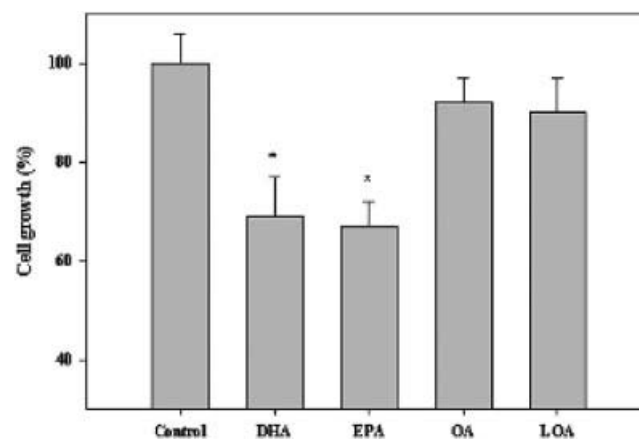
Tumor tissues were further analyzed for p21 expression using semiquantitative RT-PCR. Results presented in Figure 2b demonstrate that expression of p21 mRNA was upregulated by fish oil. mRNA expression of p21 in mice maintained on the fish oil diet was induced, whereas p21 was not detected in tumor tissues of corn-oil-diet-fed mice.

#### Effects of omega-3 fatty acids on breast cancer cells in culture (in vitro studies)

We next evaluated whether the long-chain polyunsaturated omega-3 fatty acids commonly found in fish oils (DHA and EPA) can affect MDA-MB-231 breast cancer cells *in vitro*. The dietary fish oil (Menhaden, Sigma Chemical) employed in these studies was first analyzed for its fatty acid content by gas chromatography. The fish oil was shown to have 150 mg of DHA and 160 mg of EPA per g of oil (Table II). Both DHA and EPA were then tested on cultured MDA cells, where both fatty acids similarly inhibited growth in a dose-dependent manner (Fig. 3). At concentrations as low as 25  $\mu$ M, both fatty acids inhibited cell growth by approximately 25–30% ( $p < 0.05$ ), and the inhibitory effects of



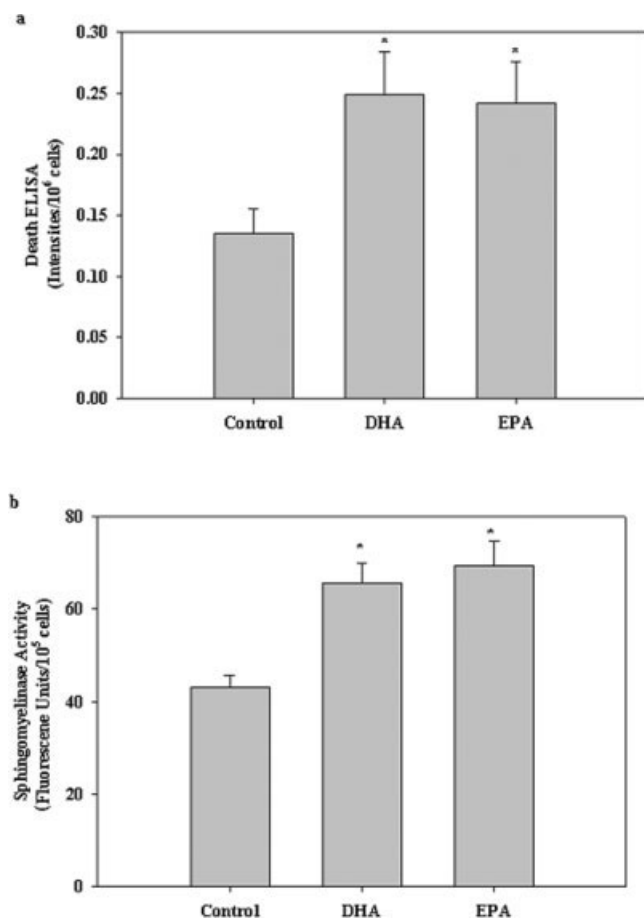
**FIGURE 3** – Dose-dependent effect of omega-3 fatty acids on breast cancer cell growth. Cells ( $1 \times 10^4$  per well) were seeded in a 96-well plate overnight and then treated with varying concentrations of DHA or EPA in serum-free medium for 24 hr. Cell growth was assayed using a WST-1 assay as described in Material and Methods. Results are the mean  $\pm$  SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the corn oil-fed group are reported (\* $p < 0.05$ ).



**FIGURE 4** – Effect of different fatty acids on cell growth. MDA-MB-231 cells were treated with 25  $\mu$ M concentrations of DHA, EPA, oleic acid (OA) or linoleic acid (LOA) and cell growth was assayed as described in Figure 4. Results are the mean  $\pm$  SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the control are reported (\* $p < 0.05$ ).

DHA and EPA progressively increased with increasing concentration to a maximum inhibition at 80  $\mu$ M by approximately 80% ( $p < 0.05$ ) after 24 hr of incubation.

We further tested whether other long-chain unsaturated fatty acids had effects similar to DHA and EPA on breast cancer growth. Results shown in Figure 4 demonstrate that at a concentration where DHA and EPA significantly inhibited MDA growth (25  $\mu$ M), oleic acid, an omega-9 fatty acid, and linoleic acid, an omega-6 fatty acid, had only a minimal effect on growth. However, a concentration  $> 100 \mu$ M of OA or LA resulted in a similar effect as of DHA or EPA at 25  $\mu$ M. Therefore, at lower concentrations, inhibition of cancer cell growth was not a general consequence of any long-chain fatty acid but rather is unique to the long-chain polyunsaturated omega-3 fatty acids, whereas at higher concentrations all fatty acids are generally cytotoxic (detergent effect).

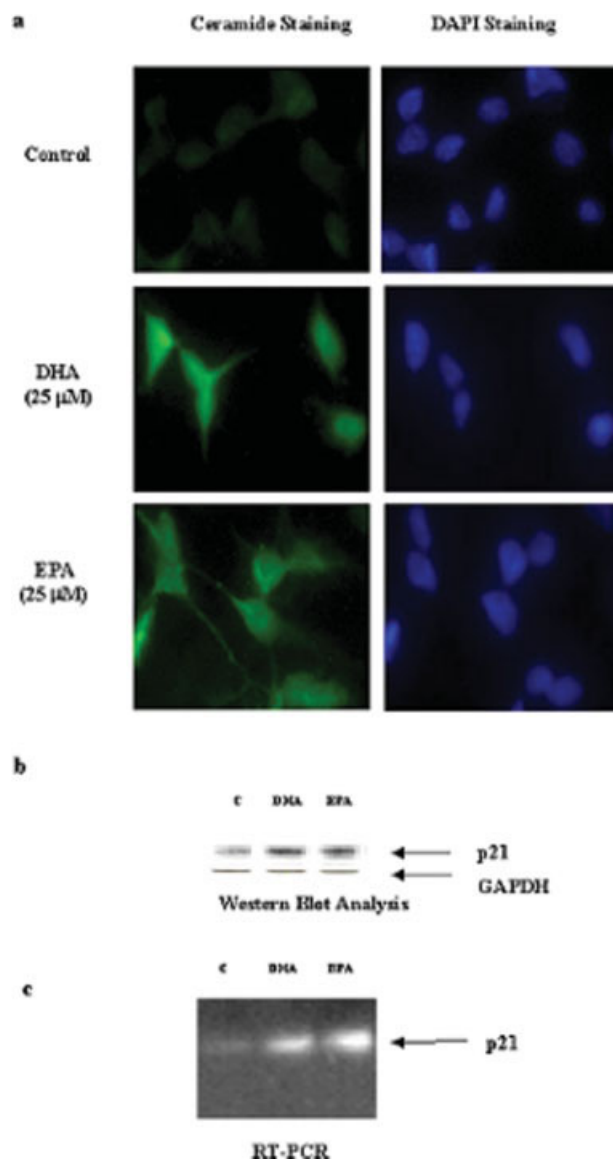


**FIGURE 5** – Effects of omega-3 fatty acids on apoptosis and N-SMYase activity in breast cancer cells. MDA-MB-231 cells ( $1 \times 10^6$ /ml) were grown in 6-well plates and treated with 25  $\mu$ M DHA or EPA as described in Figure 4. Apoptosis (a) was assayed using a cell death ELISA kit, whereas N-SMYase activity (b) was assayed using an Amplex Red kit described in Material and Methods. Results are the mean  $\pm$  SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the control are reported (\* $p < 0.05$ ).

We further examined whether growth inhibition of MDA cells was due to induction of apoptosis. Results presented in Figure 5a demonstrate that both DHA and EPA at 25  $\mu$ M induce apoptosis by 70–75% ( $p < 0.05$ ) as is evident from enhanced DNA fragmentation. Furthermore, N-SMYase activity was increased by 30–40% ( $p < 0.05$ ) in cells treated with DHA or EPA compared to those of untreated (control) cells (Fig. 5b). The increase in N-SMYase activity induced by the omega-3 fatty acids was further analyzed by assaying ceramide formation, the product of sphingomyelin hydrolysis. The noticeable generation of ceramide was observed in MDA cells upon DHA or EPA treatment (Fig. 6a) compared to that of control cells. We also analyzed the effect of DHA and EPA on p21 expression by both RT-PCR and by Western analysis. Results presented in Figure 6b indicate that expression of p21 protein was increased approximately 2.5–3-fold ( $p < 0.05$ ) in DHA- or EPA-treated cells compared to control cells. Similarly, DHA and EPA also increased expression of p21 mRNA in the same cells (Fig. 6c).

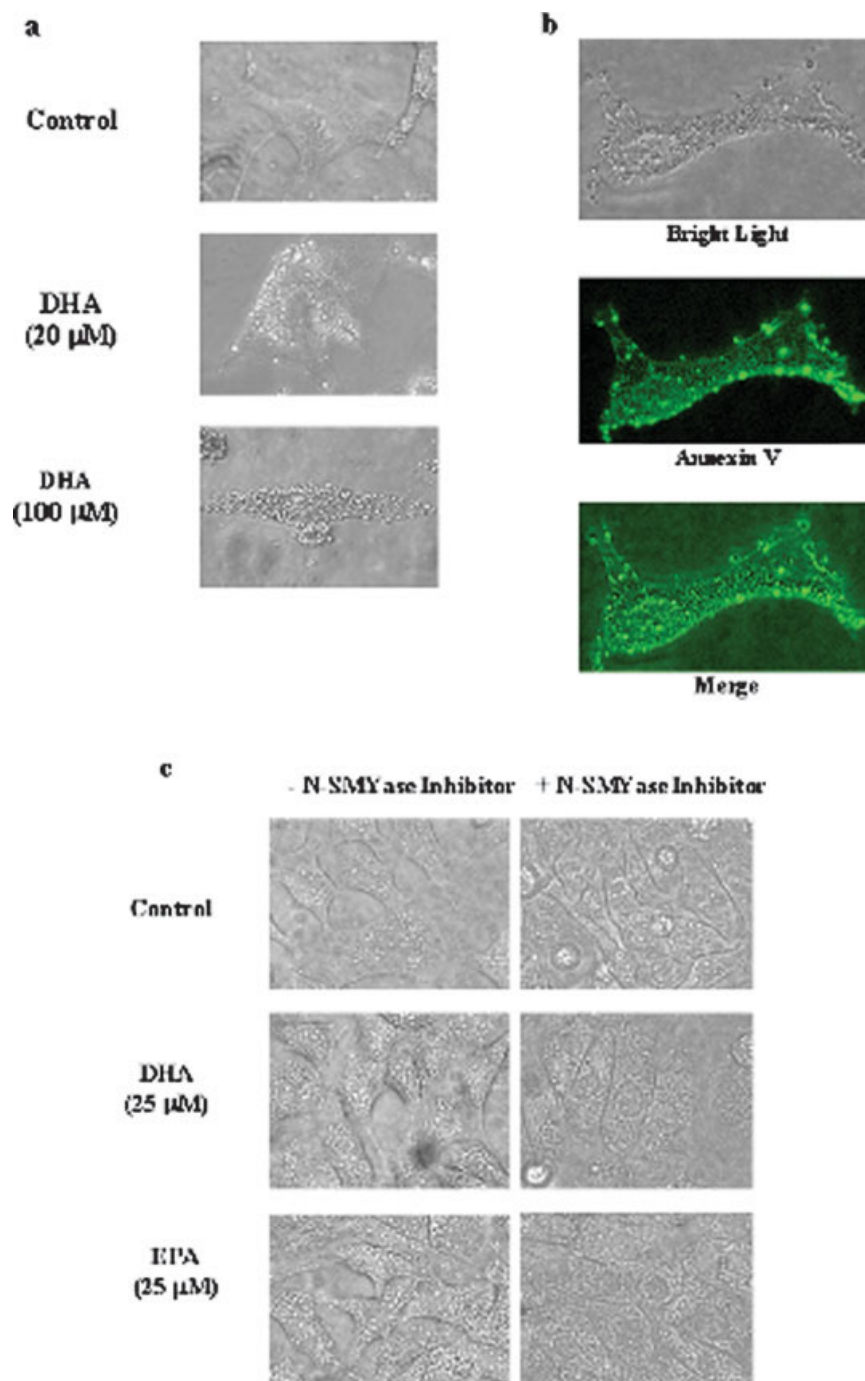
#### Effect of omega-3 fatty acids on membrane structure

One of the noticeable effects of DHA on MDA cells was the induction of plasma membrane blebs. Results shown in Figure 7a



**FIGURE 6** – Omega-3 fatty acids induce ceramide formation and p21 expression in breast cancer cells. MDA-MB-231 cells ( $1 \times 10^4$ /ml grown in 4-well chamber slides or  $1 \times 10^6$ /ml grown in T75 flasks) were treated with 25  $\mu$ M DHA or EPA as described in Figure 4. Generation of ceramide (a) was determined immunohistochemically using an anticardiolipin antibody (green fluorescence) as described in Material and Methods. Blue DAPI staining was used to visualize nuclei. Expression of p21 protein was analyzed by Western analysis (b) using GAPDH as a loading control as described in Material and Methods, whereas expression of p21 mRNA was analyzed by RT-PCR (c) as described in Figure 3. Results are representative of 3 experiments in each section.

demonstrate substantial changes in appearance (bleb formation) of the MDA cell surface caused by increasing concentrations (0–100  $\mu$ M) of DHA. The MDA cells were then stained with annexin V for the presence of externalized phosphatidylserine. Figure 7b shows that cells incubated in 50  $\mu$ M DHA demonstrated extensive bleb formation, and these blebs appear to have aggregated phosphatidylserine on the surface (the membrane blebs and annexin V are colocalized). Similar effects on membrane bleb formation were also observed upon treatment with EPA (data not shown). In addition, bleb formation appears to be related to the DHA-induced N-SMYase activity reported in Figure 5b. Results presented in



**FIGURE 7** – Effect of omega-3 fatty acids on membrane bleb formation. MDA-MB-231 cells ( $1 \times 10^4$ /ml) were grown in 4-well chamber slides and treated with varying concentrations of DHA as described in Figure 2. Cells were observed under a microscope using  $400\times$  magnification (*a*). The blebs were assayed for externalized phosphatidylserine in cells treated with  $50 \mu$ M DHA by using an annexin V binding kit as described in Material and Methods (*b*). Inhibition of omega-3 fatty acid-induced membrane bleb formation was observed in the presence or absence of  $20 \mu$ M GW4869, a N-SMYase inhibitor (*c*). Cells were observed under the microscope using  $20\times$  magnification. Results are representative of 3 experiments in each section.

Figure 7c indicate that the DHA-induced membrane bleb formation was inhibited by the N-SMYase inhibitor GW4869.

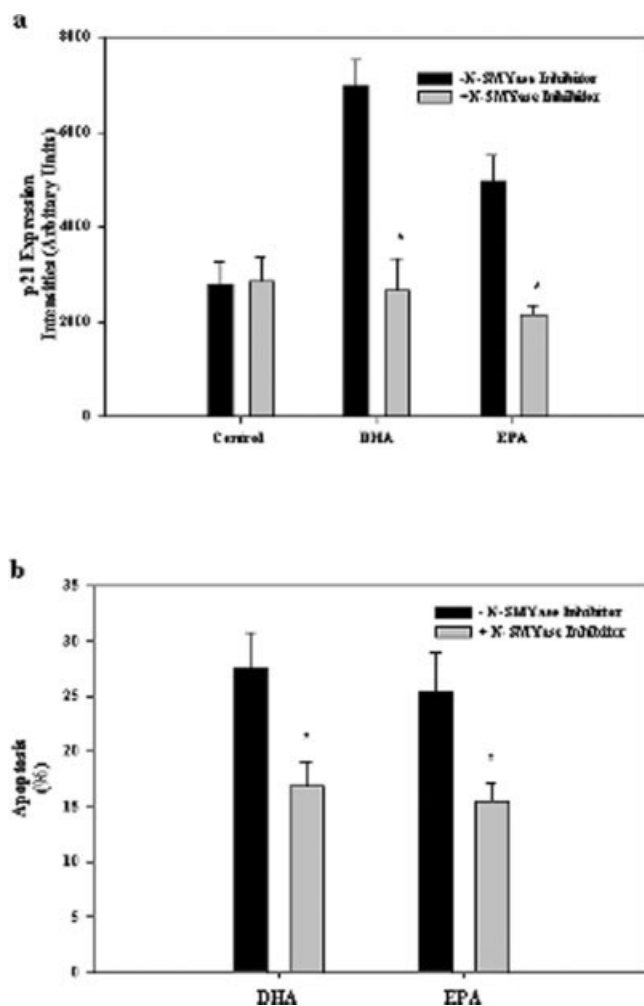
#### *Inhibition of omega-3 fatty acid-induced p21 expression and apoptosis by N-SMYase inhibitor*

The involvement of N-SMYase in DHA- or EPA-induced p21 expression was further investigated using immunohistochemistry. Figure 8a indicates that expression of p21 protein was induced in DHA- and EPA-treated cells as is evident by enhanced fluorescence intensities. This DHA- or EPA-induced expression of p21 was markedly diminished by approximately 50–60% in the presence of N-SMYase inhibitor.

Involvement of N-SMYase in DHA- or EPA-induced apoptosis was also investigated. Data depicted in Figure 8b indicate that in the presence of the N-SMYase inhibitor, DHA- and EPA-induced apoptosis in MDA cells was inhibited by approximately 40–50% ( $p < 0.05$ ).

#### **Discussion**

Breast cancer is one of the most frequently diagnosed nonskin cancers and the second most common cause of cancer death in women.<sup>42</sup> An estimated 215,990 new cases of breast cancer were expected in 2004. Epidemiologic evidence links fish oil consump-



**FIGURE 8** – Inhibition of p21 expression and apoptosis by the N-SMYase inhibitor. (a) MDA-MB-231 cells ( $1 \times 10^4$ /ml) were grown in 4-well chamber slides and then treated with 25  $\mu$ M DHA or EPA for 24 hr in the presence or absence of 20  $\mu$ M GW4869. Cells were then fixed and expression of p21 was detected using immunohistochemical methods as described in Material and Methods. Expression of p21 was quantified by densitometric analysis using a KODAK Image Station 2000MM (Eastman Kodak Company, Rochester, NY). (b) MDA-MB-231 cells in 6-well plates were incubated for 24 hr with 25  $\mu$ M DHA or EPA in the presence or absence of 20  $\mu$ M GW4869. Plates were centrifuged at 800g in a Beckman J series centrifuge to deposit floating cells at the bottom for analysis, and the supernatant was carefully removed. Quantitative analysis of apoptosis was performed by using an annexin V staining kit as described in Material and Methods. The dead and necrotic cells exhibit red fluorescence, whereas apoptotic cells fluoresce green. The total and apoptotic cells were counted and the percentage of cells exhibiting apoptosis was calculated. Results are the mean  $\pm$  SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences between groups are reported (\* $p < 0.05$ ).

tion (rich in the omega-3 fatty acids DHA and EPA) with a low incidence of several types of cancer.<sup>1–4</sup> The anticancer role of omega-3 fatty acids has also been substantiated with dietary studies using many types of animals (including humans) and numerous different cell lines, including breast cancer.<sup>8,10,19,20</sup> In the study reported here, we investigated the effects of long-chain polyunsaturated omega-3 fatty acids on breast cancer cells both *in vivo* and *in vitro*.

Our *in vivo* studies, shown in Figures 1 and 2 and Table III, demonstrate that increasing the ratio of omega-3 to omega-6 fatty

acids in the diet inhibits development of transplanted breast cancer cells in nude mice. Similar animal models to study the growth of transplanted breast cancer cells have been widely described.<sup>5</sup> The oils used for preparing the diets were examined for lipid peroxidation products as previously described<sup>24</sup> and tested for apoptosis on breast cancer cells. As previously reported for Jurkat cells, our data indicate that levels of lipid peroxidation products in oils did not correlate with the extent of apoptosis. It therefore appears that the oils but not the oxidized products are responsible for the cytotoxic effects in breast cancer cells. The purpose of our study was to link the omega-3 fatty acids abundant in dietary fish oil (DHA and EPA) to inhibition of breast cancer cell proliferation *in vivo* and to investigate one possible mode of action, namely the effect on N-SMYase.

Our studies were then extended to cultured MDA-MB-231 cells, in which we initially investigated whether the constituents of fish oil—DHA and EPA—could inhibit breast cancer proliferation *in vitro*. Data presented in Figure 3 indicate that both DHA and EPA were equally effective in inhibiting MDA-MB-231 cell growth. Similar inhibition was not observed with either oleic acid, an omega-9 fatty acid that is the most abundant fatty acid in animal tissues, or linoleic acid, an omega-6 fatty acid abundant in common vegetable oils (Fig. 4), indicating that inhibition is not just a general property of all long-chain fatty acids. The data presented in Figure 5a indicate that inhibition of cancer cell growth was likely due to induction of apoptosis by the omega-3 fatty acids.

We further evaluated a possible signaling pathway that may be responsible for regulating tumor growth by omega-3 fatty acids. Our previous studies suggest that DHA inhibits the growth of Jurkat leukemic cells partially through activation of N-SMYase, increased ceramide formation and enhanced expression of p21.<sup>23</sup> To link the breast cancer studies to our previous work with Jurkat leukemic cells, we monitored the activity of N-SMYase and p21 expression both *in vivo* (nude mice) and *in vitro* (cultured MDA-MB-231 cells). Results shown in Figure 2 demonstrate that tumors from mice fed a corn oil (omega-6)-based diet have considerably lower N-SMYase activity than mice fed on diets containing significantly higher omega-3 fatty acid levels. Similarly, treatment of cultured MDA-MB-231 cells with DHA or EPA enhanced N-SMYase activity (Fig. 5b), resulting in increased ceramide formation (Fig. 6a) and enhanced p21 expression as analyzed by Western blot and immunohistochemical analysis (Fig. 6b) and confirmed by RT-PCR (Fig. 6c). RT-PCR in our present study was performed as an extra measure to confirm increased expression of p21 in the presence of DHA or EPA. Although products generated after 30 cycles do not indicate a quantitative measure of p21 mRNA expression in these cells, they clearly demonstrate differences between control and treated cells.

Ceramide-induced activation of cellular N-SMYase-mediated signaling pathways in response to stress has been previously reported by Hannun *et al.*<sup>43</sup> and Kolesnick *et al.*<sup>44</sup> The N-SMYase pathway is known to be activated by various factors including heat, ischemia/reperfusion, oxidants, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), Fas ligand, vitamin D3, IL-1 and  $\alpha$ -interferon and initiates cellular events leading to cell death or apoptosis.<sup>45,46</sup> Hydrolysis of SM by N-SMYase generates ceramide, a lipid that is regarded as a “universal component of apoptosis.”<sup>47–49</sup> About 70% of cellular SM is present in the outer leaflet of plasma membranes, primarily in lipid rafts, where it probably serves to stabilize rafts by interacting with cholesterol and phospholipids containing saturated fatty acids.<sup>50,51</sup> Several studies suggest that N-SMYase is generally localized in plasma membranes.<sup>32,52</sup> Activation of cells by TNF has been shown to induce translocation of N-SMYase from detergent-resistant membrane fractions (lipid rafts) to detergent-soluble (nonraft) fractions, resulting in enhanced activity.<sup>34</sup> In a preliminary study (data not shown), we have also demonstrated that DHA treatment decreases N-SMYase activity in the detergent-resistant (raft) fractions, whereas it increases activity in the detergent-soluble (nonraft) fractions. These results suggest that omega-3 fatty acids induce changes in

the plasma membrane composition and structure of MDA-MD-231 cells, affecting N-SMYase activity by either translocating the enzyme from detergent-resistant to detergent-soluble fractions or by directly affecting N-SMYase activity (conformational change) in these fractions. Although interesting, these results need to be verified using specific antibodies against N-SMYase, which are under development in our laboratory.

We have previously demonstrated that DHA causes substantial changes in the domain structure of model membranes and isolated plasma membranes<sup>35</sup> and that incorporation of DHA instigates cellular changes leading to apoptosis.<sup>23</sup> The experiments reported here further suggest that the addition of DHA causes visible changes in the plasma membrane as indicated by the appearance of membrane blebs with externally exposed PS (annexin V binding) (Fig. 7a,b). Furthermore, DHA-induced membrane bleb formation and p21 expression was inhibited by the N-SMYase inhibitor GW4869, indicating that ceramide generation is involved in this process. In fact, blebbing and externalization of PS are hallmarks of the execution phase of apoptosis<sup>53</sup> and are believed to be

related to ceramide generation. In agreement with these findings, we observed that the N-SMYase inhibitor also reduced DHA- and EPA-induced apoptosis (Figure 8).

In conclusion, the data presented here strongly indicate a relationship between the omega-3 fatty acids DHA and EPA, tumor growth suppression, membrane structure, N-SMYase activity, ceramide formation, p21 expression and apoptosis. Our results suggest that modulation of the N-SMYase-ceramide pathway represents a potential pathway for treatment of breast cancer.

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## **ABSTRACTS**

### **PART II**

Abstracts 568.1 – 1024.3

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## WOMEN SCIENTISTS' NETWORKING AND MENTORING SESSION/RECEPTION (953.1)

953.1

### Women Scientists' Mentoring/Networking Session

Adele J Wolfson<sup>1</sup>, Marilee Benore-Parsons<sup>2</sup>. <sup>1</sup>Chemistry Department, Wellesley College, 106 Central Street, Wellesley, MA, 02481, <sup>2</sup>Department of Natural Sciences, Univ Michigan Dearborn, 4901 Evergreen Road, Dearborn, MI, 48128

This panel and reception have become a regular feature of the annual meeting. The session is intended as a forum for discussion of personal histories and strategies for increasing access for women in biochemistry. This year's session will focus on "Programs that Work," proven successes in promoting women in the sciences. Programs that support women in gaining admission to graduate school, in entering the faculty ranks, and in advancing to leadership positions will be discussed. The reception following the panel will provide opportunities for forming mentoring partnerships.

## MOLECULAR TARGETS IN DIET AND CANCER II (965.1-965.8)

965.1

### Inhibitory Effect of Prolonged-Butyrate Treatment on Migration and Invasion of HT1080 Tumor Cells

Huawei Zeng, Mary Briske-Anderson GF Hum Nutr Res Ctr, USDA, 2420 2nd Ave No, PO Box 9034, Grand Forks, North Dakota, 58202-9034

Butyrate, a normal constituent of the colonic luminal contents, has been hypothesized that butyrate may inhibit the invasion of tumor cells. The present study was to investigate the effects of butyrate on the growth, migration, and invasion characteristics of tumor HT1080 cells. HT1080 cells cultured in the presence of 0.5 and 1 mmol/L butyrate for 14 d exhibited an increase in the G1 and G2 fractions with a concomitant drop in the S-phase, thus showing slower cell growth. Interestingly, 0.5 and 1 mmol/L butyrate inhibited the migration and invasion rate of the tumor cells when compared with the untreated cells. The protein and mRNA levels of the tissue inhibitors of metalloproteinase-1 (TIMP-1) and TIMP-2 were significantly increased in HT1080 cells cultured with 0.5 and 1 mmol/L butyrate. Enzymatic activities and the mRNA level of the latent forms of matrix metalloproteinase (MMP), pro-MMP-2 and pro-MMP-9, were also increased in HT1080 cells cultured with 0.5 and 1 mmol/L butyrate. In contrast, the active MMP-2 was detectable by zymographic analysis in control but not butyrate conditioned media. Collectively, these results demonstrate that prolonged and low-dose butyrate treatment increases both pro-metastasis MMP-2, -9 and anti-metastasis TIMP-1, -2 expression, and the net effect of these increases is the inhibition of pro-MMP-2 activation and of tumor cell migration/invasion potential.

965.2

### Omega-3 fatty acids attenuate breast cancer growth through activation of a sphingomyelinase-mediated pathway

Rafat Siddiqui<sup>1</sup>, Min Wu<sup>1</sup>, Kevin Harvey<sup>1</sup>, William Stillwell<sup>2</sup>, Gary Zaloga<sup>1</sup>. <sup>1</sup>Methodist Research Institute, 1801 N. Capitol Ave, Indianapolis, IN, 46206, <sup>2</sup>Indiana University-Purdue University, 723 W. Michigan Street, Indianapolis, IN, 46202

The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. *In Vivo Experiments*: Mice were fed diets that were either omega-3-rich (fish oil) or omega-6-rich (corn oil). Three weeks post implantation, the tumor volume and weight decreased significantly ( $P < 0.05$ ) for mice fed the omega-3 diets compared to those fed the omega-6 diets. Fish oil also caused increased neutral sphingomyelinase (N-SMYase) activity by 40% ( $P < 0.05$ ) in the tumors. *In Vitro Experiments*: DHA and EPA inhibited growth of MDA-MB-231 cells in culture in a dose dependent manner. A concentration as low as 25  $\mu$ M, DHA and EPA inhibited the growth by up to 20-25% ( $P < 0.05$ ). N-SMYase activity was also increased by 70-75% ( $P < 0.05$ ) in these cells. In addition, increased ceramide formation was observed in MDA-231-MB cultured cells upon DHA or EPA treatment. Upon fractionation of tumor cell membranes, DHA was shown to enhance N-SMYase activity in the detergent-soluble (non-raft)

fractions. DHA and EPA were both observed to enhance membrane bleb formation and also induce the expression of p53 and p21. Blebs were shown to exhibit outer leaflet phosphatidylserine as assayed by annexin V staining. DHA- or EPA-induced bleb formation and apoptosis was inhibited by 40% ( $P < 0.05$ ) in the presence of the N-SMYase inhibitor (GW4869). In conclusion, our results suggest that inhibition of breast cancer growth in nude mice by fish oil or inhibition of breast cancer cell growth in culture by treatment with DHA and EPA appear to be mediated by generation of ceramide through enhanced N-SMYase activity.

965.3

### The Green Tea Compound EGCG Regulates Wnt Signaling through the HBP1 Transcriptional Repressor

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Dysregulation of Wnt signaling has been observed in numerous cancers. In breast cancer, excessive beta-catenin levels are associated with poor prognosis. Cyclin D1, Cox-2, c-MYC and other cancer genes are increased with de-regulated Wnt signaling. Our previous work has identified the HBP1, as a suppressor of Wnt signaling, a repressor of these genes, and an inhibitor of G1 progression. Ongoing work has linked HBP1 to suppression of invasive breast cancer. We reasoned suppressing Wnt signaling might be important for tumor suppression and for cancer prevention. Using the suppression of Wnt signaling as criteria for a screen of phytonutrients, the green tea compound EGCG ((-)-epigallocatechin-3-gallate) was the best candidate. EGCG has been linked to a reduced risk of cancer in animal models and in human breast cancer.

In breast cancer cells, Wnt signaling was inhibited by EGCG in a dose-dependent manner. While the levels of beta catenin were unchanged, the levels of HBP1 were increased with EGCG. DNA-based siRNA was used to knockdown the HBP1 protein, and to assess the consequences for both Wnt signaling and for EGCG suppression. As expected, the knockdown of HBP1 conferred increased sensitivity to Wnt signaling, and higher target gene expression. The HBP1 siRNA breast cell lines had reduced sensitivity to EGCG in the suppression of Wnt signaling. In addition, EGCG reduced the proliferation and invasiveness that characterizes invasive breast cancer. Together, these data suggest that EGCG inhibits Wnt signaling by inducing the HBP1, which could be a potential new biomarker in future prevention studies for invasive breast cancer (supported by NIH and DOD).

965.4

### Luteolin inhibits cell proliferation by inducing cell cycle arrest in G1 and G2/M phases in HT-29 human colon cancer cells

Do Y. Lim, Jung H Y Park. Life Sciences, Hallym University, Division of Life Sciences, Hallym University, Chunchon, 200-702, Korea, Republic of

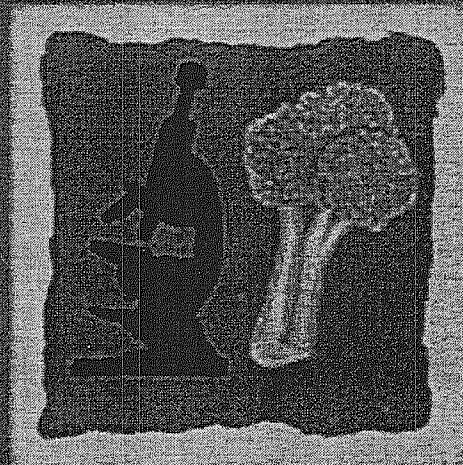
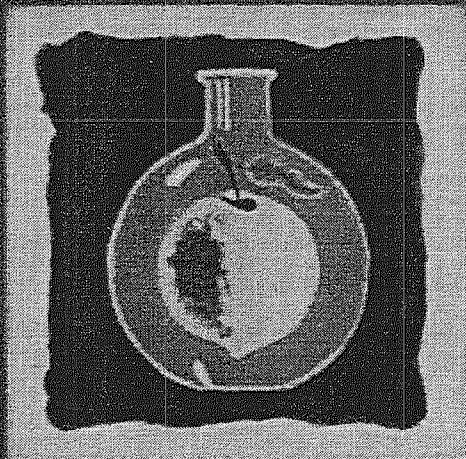
Luteolin is a 3',4',5,7-tetrahydroxyflavone found in celery, green pepper and perilla leaf. It has been found to exhibit antimutagenic, antitumorigenic, antioxidant and anti-inflammatory properties. To examine the effect of luteolin on HT-29 cell growth, cells were cultured in the absence or presence of various concentrations of luteolin. Luteolin decreased a viable HT-29 cell number in a concentration dependent manner. The decrease in cell growth was due to an increase in apoptosis and a decrease in DNA synthesis. A G1 phase arrest was induced within 2 h after addition of 60  $\mu$ M luteolin. Luteolin decreased phosphorylated retinoblastoma proteins (Rb) and cyclin D1, and increased hypophosphorylated Rb levels. However, luteolin did not alter cyclin A, cyclin E, cyclin-dependent kinase (CDK) 2 or 4 levels. The activities of CDK2 and CDK4 were decreased by luteolin in a time dependent manner. A dose-dependent decrease in CDK4 activity was observed within 2 h after the addition of luteolin which correlated with the decrease in cyclin D1 levels. Luteolin also arrested cell cycle progression at G2/M phase following 24 h after the luteolin treatment. Luteolin treatment did not alter phospho-cell division cycle (CDC)2, total CDC2 or phospho-cyclin B1 protein levels. However, cyclin B1 and cdc25C were dose-dependently decreased in concert with increased CDC2 activity. We have demonstrated that luteolin decreases the HT-29 cell growth by increasing apoptosis and arresting cell cycle progression

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**Neutral sphingomyelinase mediates inhibitory effects of omega-3 polyunsaturation on breast cancer development.** Rafat A. Siddiqui<sup>1,2,3</sup>, Min Wu<sup>1</sup>, Nargiz Ruzmetov<sup>1</sup>, Kevin A. Harvey<sup>1</sup>, Zachary R. Welch<sup>1</sup>, Laura Sech<sup>1</sup>, Kim Jackson<sup>2</sup>, Gary P. Zaloga<sup>1,3</sup>, and William Stillwell<sup>2</sup>, <sup>1</sup>Cellular Biochemistry Laboratory, Methodist Research Institute, Clarian Health Partners, Indianapolis; <sup>2</sup>Department of Biology, Indiana University-Purdue University, Indianapolis; and <sup>3</sup>Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana.

## ABSTRACT

The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. *In Vitro* Experiments: DHA and EPA inhibited the growth of cultured MDA-MB-231 cells in a dose-dependent manner ( $P < 0.05$ ). Neutral sphingomyelinase (N-SMYase) activity was also increased 30-40% ( $P < 0.05$ ) in the DHA or EPA treated cells where an increase in ceramide formation was observed. DHA and EPA both enhanced membrane bleb formation and also induced the expression of p21. Both bleb formation and p21 expression were inhibited by the N-SMYase inhibitor GW4869, which also inhibited apoptosis by ~40% ( $P < 0.05$ ). *In Vivo* Experiments. Mice were fed diets that were rich in either omega-3 (fish oil) or omega-6 (corn oil) fatty acids. Three weeks after implantation of MDA-MB-231 breast cancer cells, tumor volume and weight were significantly lower ( $P < 0.05$ ) for mice fed the omega-3 diet compared to those fed the omega-6 diet. Dietary fish oil also caused a 40% ( $P < 0.05$ ) increase in N-SMYase activity in the tumors. The tumor tissues from fish oil-fed animals expressed elevated p21 mRNA, whereas tumor tissues from corn oil-fed did not. DHA and EPA also caused a translocation of N-SMYase activity from plasma membranes to intracellular sites. The results suggest that inhibition of breast cancer cell growth in culture by treatment with DHA or EPA and inhibition of breast cancer growth in nude mice by dietary fish oil is mediated by N-SMYase translocation from membranes to intracellular sites with subsequent enhancement in N-SMYase activity.

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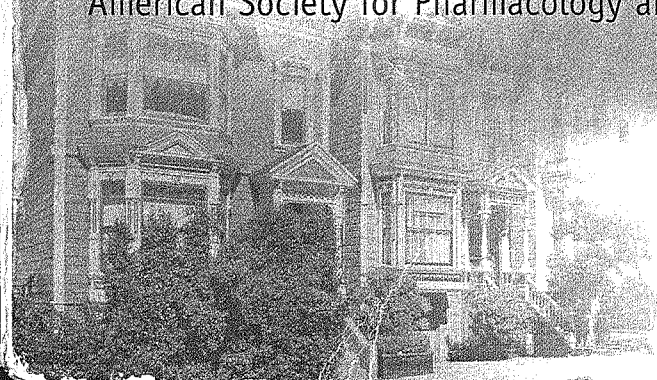
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**Docosahexaenoic acid (DHA) and polyphenols synergistically induce apoptosis in breast cancer cells by activating protein phosphatases**

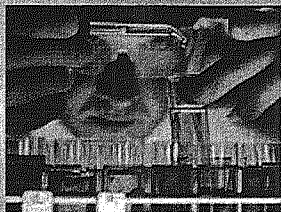
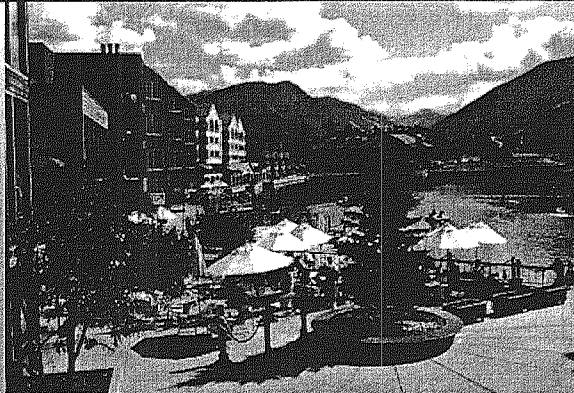
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We have investigated the ability of omega-3 fatty acids combined with dietary polyphenols to induce apoptosis in breast cancer cells. The objective of the present study was to investigate the protein tyrosine-mediated and protein serine/threonine kinase-mediated pathways to assess the anticancer effects of these molecules. MDA-MB-231 cells were treated with various concentrations of polyphenols (Curcumin, Resveratrol, Myricetin, Quercetin, Green Tea Polyphenols and Grape Skin Extracts) in the absence or presence of DHA (25  $\mu$ M). Effects on cell viability were measured using WST-1 and LDH assays. Induction of apoptosis was analyzed using a vibrant apoptotic assay kit. Phosphorylation of cellular proteins was analyzed using phosphoserine- and phosphotyrosine-specific antibodies. Results of these studies demonstrate that polyphenols act synergistically with DHA to induce apoptosis in breast cancer cells. All of the polyphenols and omega-3 fatty acids tested inhibited tyrosine and serine/threonine phosphorylation of several proteins in breast cancer cells in a dose-dependent manner. However, these agents stimulated serine phosphorylation of a protein at 38 kDa, which was identified using Western analysis as p38 kinase, a member of MAP-kinase super-family. Our data indicate that dietary polyphenols and omega-3 fatty acids may inhibit breast cancer growth by activating cellular protein phosphatases, which induces downstream phosphorylation of Thr 180/Tyr 182 residues on p38 kinase, a regulatory protein involved in cell apoptosis.

# Abstracts

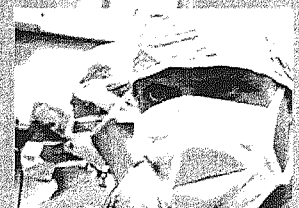
## 2nd Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences — Targeting Heart Failure: New Science, New Tools, New Strategies

July 24–27, 2005  
Keystone Conference Center  
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Sponsored by the American Heart Association Council on Basic Cardiovascular Sciences

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by two structurally independent inhibitors of the proteasome system, clasto-lactacystin  $\beta$ -lactone and MG132. Selective activation of PKC $\epsilon$  in cardiac cells enhanced PKC $\epsilon$  phosphorylation and reduced PKC $\epsilon$  protein degradation, demonstrating that phosphorylation/activation of PKC $\epsilon$  renders this kinase less susceptible to degradation. This effect of phosphorylation on proteasome dependent degradation was also confirmed by phosphorylation site mutagenesis studies on PKC $\epsilon$ . The half-life of PKC $\epsilon$  and PKC $\epsilon$  T566A (a mutation which prevents phosphorylation of this residue) in Cos7 cells was determined by pulse-chase experiments to be  $\sim 22$  and  $\sim 8$  hours respectively. Furthermore, cardiac tissues from PKC $\epsilon$  transgenic mice exhibiting a cardioprotective phenotype showed increased PKC $\epsilon$  activity, elevated PKC $\epsilon$  phosphorylation, decreased PKC $\epsilon$  ubiquitination, and reduced PKC $\epsilon$  degradation compared with those in wild type control mice. In contrast, cardiac tissue samples from transgenic mice expressing dominant negative PKC $\epsilon$  displayed reduced PKC $\epsilon$  activity, attenuated PKC $\epsilon$  phosphorylation, and enhanced PKC $\epsilon$  ubiquitination. Taken together, these data demonstrate (i) that the ubiquitin-proteasome system modulates PKC $\epsilon$  degradation in cardiac cells and (ii) that activation of PKC $\epsilon$  in cardioprotection attenuates the degradation of this kinase, suggesting a possible critical role of the UPS in sustained activation of protective proteins and thereby cardioprotection.

**M. Lu**, None; **A.V. Gomes**, None; **C. Zong**, None; **B.T. Berhane**, None; **D. Pantaleon**, None; **X. Qiao**, None; **G. Wang**, None; **P. Ping**, None.

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#### Redox Regulation of Protein Kinase C in Myocardial Ischemia/Reperfusion

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Protein kinase C (PKC), a key signaling kinase, is one of those subjected to redox control representing a new paradigm of the alternate signaling principle. We were among the first to establish that ROS directly act on PKC, releasing chelated zinc ions from the zinc finger of the regulatory domain. Our studies led to the unexpected and intriguing result suggesting that in addition to serving a structural function, zinc ions are likely to play a dynamic regulatory role. The evidence obtained by us clearly defines cysteine-rich domain as a redox sensor and a reversible redox switch. We extend our findings to the whole heart model and now demonstrate physiological implication of zinc movements during myocardial ischemia/reperfusion. **Methods:** Langendorff perfused adult rat and mouse hearts were subjected to 15 min global ischemia followed by 20 min reperfusion (I/R). Free Zn $^{2+}$  in isolated cardiomyocytes and cryo-sections was assessed by confocal microscopy using TSQ as a probe. **Results:** TSQ fluorescence in cryo-sections originated from sarcomeric units, cell periphery, and intercalated disks similar to the pattern observed in isolated cells. Oxidatively triggered Zn $^{2+}$  release was reversed by reduction with N-acetylcysteine. Areas with irregular morphology were observed in I/R tissue sections. TSQ decorated zones of cell contacts, as well as patchy areas containing granulated vesicle-like structures. Fascinatingly, overall fluorescence intensity was much lower in I/R stressed heart compared to control. PMA treatment significantly increased TSQ fluorescence of tissue sections obtained from control hearts (90% increase) but not from the ones after I/R (14% increase). The transgenic mouse model with altered function of PKC $\epsilon$  revealed preservation of zinc response by activated kinase. **Conclusions:** Being an integrated composite of redox signaling systems, free zinc reflects the protein redox status and serves a valid biomarker of stressed tissue and its capacity to respond to stimuli. Our approach to investigate functional zinc and its movements in situ would apply to human myocardial tissues samples. We believe that setting up the protein redox switch in accordance to the data from those samples would provide clues to individual approach to cardioprotection.

**M. Boutjdir**, None; **Y. Yue**, None; **I. Korichneva**, None.

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#### Functional Significance of a New PKC- $\alpha$ Phosphorylation Site on Inhibitor-1 of Cardiac Protein Phosphatase-1

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Protein phosphatase 1 (PP1) plays a pivotal role in the development of heart failure. PP1 is regulated in vivo by inhibitor-1 (I-1). When I-1 is phosphorylated at Thr-35 by PKA it inhibits PP1. However, recent studies reported that I-1 can be also phosphorylated by PKC- $\alpha$  on Ser-67 and suggested that this may decrease the ability of I-1 to inhibit PP1. Given that both PKC- $\alpha$  and PP1 activities are significantly increased in failing hearts, we further examined PKC- $\alpha$

mediated phosphorylation of I-1, using purified proteins. We cloned and expressed cDNAs, encoding human I-1 or an I-1 mutant with alanine substitution at Ser-67. The obtained recombinant proteins were purified and the GST-tag was removed. PKC- $\alpha$  phosphorylation of the pure proteins indicated that 32P-incorporation into the mutant was decreased but not completely abolished in comparison to the I-1 wild type, suggesting that there may be another PKC- $\alpha$  phosphorylation site. For identification of this putative PKC- $\alpha$  site, phosphorylated human I-1 was subjected to matrix-assisted laser desorption ionization mass spectrometry in combination with Edman degradation. These analyses revealed threonine-75 as a new PKC- $\alpha$  site on human I-1. To confirm these data, I-1 mutants with alanine substitutions at Thr-75 (T75A), and Ser-67 plus Thr-75 (S67A / T75A) were generated. PKC- $\alpha$  treatment of I-1 and its mutants showed reduced 32P-incorporation into either S67A or T75A and none in the S67A / T75A mutant. Further analysis by two-dimensional electrophoresis corroborated that: 1) Thr-75 is a PKC- $\alpha$  site; and 2) Ser-67 and Thr-75 are the only residues phosphorylated by PKC- $\alpha$  on human I-1. To determine the functional significance of Thr-75 phosphorylation, protein phosphatase assays were performed. Phosphorylation of I-1 or I-1 mutants by PKA was associated with inhibition of PP1. However, PKC- $\alpha$  phosphorylation of I-1 had no effect on its activity. Furthermore, PKC- $\alpha$  phosphorylation had no effect on the PKA-mediated inhibitory function of I-1. These in vitro findings suggest that there exists an additional PKC- $\alpha$  site in human I-1 but its phosphorylation has no significant effect on its inhibitory activity. Future in vivo experiments may further elucidate the functional role of this new I-1 phosphorylation site.

**P. Rodriguez**, None; **B. Mitton**, None; **E. Kranias**, None.

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#### Omega-3 Fatty Acids Inhibit Protein Kinase A and Calcium Calmodulin Kinase II Activities and Improve Survival following Myocardial Infarction

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Epidemiological and clinical data suggest that omega-3 polyunsaturated long chain fatty acids (n-3 PUFAs) decrease sudden death in patients with coronary artery disease following myocardial infarction. However, the mechanisms for the beneficial effects of n-3 PUFAs are unknown. The objectives of the present study were to confirm the findings from clinical trials using an animal model of myocardial infarction in which dietary intake could be closely controlled and to utilize this model to investigate molecular mechanisms for the beneficial effects of n-3 PUFAs. Using an animal model of myocardial infarction (ie. coronary ligation), we report that a diet high in n-3 PUFAs was associated with a significant improvement in six-month mortality compared with animals consuming a diet high in n-6 PUFAs [86.5% (32 out of 37) vs 64.9% (24 out of 37),  $p < 0.01$ ]. Plasma samples and cardiac tissues of animals maintained on n-6 PUFA or n-3 PUFA diets exhibited significantly elevated n-6 PUFA or n-3 PUFA levels, respectively. The improved survival was associated with decreased activities of protein kinases A and calcium-calmodulin dependent kinase II. Total kinase activity was decreased by 40% ( $n=10$ /group) in hearts from n-3 fed rats compared to those of those of n-6 fed rats. These hearts also exhibited decreased PKA and CaMKII activities by 35% ( $n=10$ /group,  $P < 0.05$ ) and by 46% ( $n=10$ /group,  $P < 0.05$ ), respectively, whereas PKC activities did not change significantly. We also found that heart-specific ryanodine receptor 2 phosphorylation was decreased in the omega-3 diet group by  $\sim 20\%$  ( $n=10$ /group,  $P < 0.05$ ) compared to those of n-6 animals. Therefore, we conclude that decreased activity of protein kinases induced by diets high in n-3 PUFAs contributes to the decrease in sudden death following myocardial infarction.

**R.A. Siddiqui**, None; **N. Ruzmetov**, None; **K.A. Harvey**, None; **M. Zerouga**, None; **C. Terry**, None; **N. Patel**, None; **W. Stillwell**, None; **G.P. Zaloga**, None.

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#### AMP-Kinase Phosphorylation Correlates with Arrest Duration and Return of Spontaneous Circulation in a Murine Model of Cardiac Arrest

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Cardiac arrest accounts for approximately 300,000 deaths each year in the United States. Efforts to improve survival require an understanding of the cellular mechanisms of intra- and post-arrest